Antimicrobial Resistance in *Salmonella enterica* Typhi in Asia

Thesis

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Antimicrobial Resistance in *Salmonella enterica* Typhi in Asia

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Abstract

Antimicrobial resistance is a major problem in the treatment of typhoid fever; a systemic human infection caused by *Salmonella* Typhi (*S.* Typhi). The co-existence of multidrug resistant and fluoroquinolone resistant *S.* Typhi isolates is increasingly common. Therefore, it is essential to understand the molecular mechanisms and the epidemiology of antimicrobial resistant *S.* Typhi in endemic countries such as Viet Nam. A cross section of *S.* Typhi strains isolated from 8 Asian countries and a Vietnamese *S.* Typhi collection were investigated for antimicrobial resistance patterns. Molecular approaches were used to identify the mechanisms of (fluoro)quinolone resistance in *S.* Typhi isolates. Specific phenotypic characteristics of each of the naturally mutations were assessed in *S.* Typhi isolates that were reconstructed in an isogenic strain. The antimicrobial resistance phenotypes of both wild type and reconstructed mutants were calculated using antimicrobial susceptibility tests and time - kill experiments. To predict the relative fitness of these mutants, the strains were subjected to independent and competitive growth assays. Finally, we developed a molecular epidemiological tool which was used to genotype and identify fluoroquinolone resistant *S.* Typhi strains circulating in Asia. The findings of this study show that antimicrobial resistant *S.* Typhi is continuing problem in Asian countries, especially in Viet Nam. Fluoroquinolone resistance in *S.* Typhi is caused by mutations in specific locations of the *gyrA* and *parC* genes. The majority of reconstructed mutants had a selective growth advantage when placed in competition with a non - mutant *S.* Typhi strain. Strains that had the highest level of fluoroquinolone resistance had a selective disadvantage. We conclude that *S.* Typhi haplotype H58 with associated nalidixic acid resistance is dominant across Asian countries.

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Abbreviations

&	And
µg	Microgram
µM	Micromole
µL	Microliter
A	Alanine
AFLP	Amplified Fragment Length Polymorphism
AMP	Ampicillin
API20E	Analytical Profile Index 20 enterobacteria
ASO	Allele - Specific Oligo
ATP	Adenosine Tri-Phosphate
AZM	Azithromycin
BLAST	Basic Local Alignment Search tool
bp	Base pairs
cat	Chloramphenicol acetyl transferase
CFU	Colony Forming Unit
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CRO	Ceftriaxone
CTAB	Cetyl-Trimethylammonium Bromide
D	Aspartic acid
ddNTP	Dideoxyribonucleotide triphosphate
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTCS	Dye Terminator Cycle Sequencing
ESBL	Extended Spectrum Beta Lactamase
F	Phenylalanine
G	Glycine
g	Gram
GAT	Gatifloxacin
I	Isoleucine
ID	Identification
IR	Inverted Repeat
IVI	International Vaccine Institute
Kb	Kilobase pairs
kV	Kilovolt
L	Liter
LB	Luria Bertani media
LEX	Levofloxacin
LPS	Lipopolysaccharide
LSO	Locus - Specific Oligo
MC	MacConkey
MDR	Multi-Drug-Resistant
MH	Mueller-Hinton

MIC	Minimum inhibitory concentration
mL	Milliliter
MLPA	Multiple Ligation Dependent – Probes Amplification
MLST	Multilocus Sequence Typing
mM	Milimole
mob	DNA Mobilizations
MRD	Maximum Recovery diluents
N	Asparagine
NAL	Nalidixic acid
NCBI	National Centre for Biotechnology Information
nm	Nanometer
No	Number
NOX	Norfloxacin
OFX	Ofloxacin
ori	Origin
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
pM	Picomole
PMQR	Plasmid mediated quinolone resistance
PPi	Pyrophosphate
QRDR	Quinolone Resistance - Determining Regions
RAPD	Randomly Amplified Polymorphic DNA
rpm	Revolutions per minute
Rpt	Repeats
S	Serine
S.	*Salmonella*
SNP	Single nucleotide polymorphism
SPI	*Salmonella* pathogenicity Island
SXT	Sulfamethoxazole- Trimethoprim
TE	Tris- EDTA buffer
UV	Ultraviolet
Vi	Virulence antigen
WHO	World Health Organization
x	Time
XLD	Xylose Lysine Deoxycholate
Y	Tyrosine
µF	MicroFaraday
Ω	Ohm
°C	Degree Celsius

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1. Introduction

1.1 The genus *Salmonella*

Salmonella is genus of the bacterial family *Enterobacteriaceae*. *Enterobacteriaceae* or (more commonly) enteric bacteria generally inhabit the intestinal tract of both warm and cold blooded animals. This large family consists of genetically related bacteria, which have similar phenotypic and biochemical properties. The most closely related species to *Salmonella* is *Escherichia* and both these groups of organisms share common traits which aid survival and transmission [1]. *Salmonella* are facultative anaerobic bacilli that are gram negative and generally mobile (with the ability to swarm and swim on the basis of flagella). Microscopically they measure between 2 – 4 x 0.4 – 0.6 μm in size and are biochemically identified by the production of gas from glucose, manitol, maltose and sorbitol and the production of hydrogen sulphide [1].

1.2 Taxonomy

According to the CDC classification system, the genus of *Salmonella* is divided into 2 species, *Salmonella enterica* and *Salmonella bongori* [2]. *Salmonella enterica* is further subdivided into six subspecies that are designated by names and Roman numerals (I; *S. enterica* subsp. *enterica*, II; *S. enterica* subsp. *salamae*, IIIa; *S. enterica* subsp. *arizonae*, IIIb; *S. enterica* subsp. *diarizonae*, IV; *S. enterica* subsp. *houtenae* and VI; *S. enterica* subsp. *indica*). *S. enterica* subspecies can be differentiated by biochemical properties and by genomic relatedness. The organisms in *S. enterica* subsp. I cause approximately 99% of all *Salmonella* infections in humans and other warm-blooded animals and are the general focus of global *Salmonella* research [2].

1.3 *Salmonella* biochemical and microbiological identification

Salmonella can be cultured on blood agar and MacConkey agar media [1]. Colonies on blood agar and MacConkey agar media have a grey opaque appearance with an average diameter of 2-3 mm. The colony morphology is generally circular, with a smooth surface, or flatter with a less regular surface [1]. Currently, chromogenic agar (CHROMagar) and Xylose Lysine Deoxycholate agar (XLD) are recommended for *Salmonella* isolation [3].

Salmonella enterica serovar Typhi (all *Salmonellae* serovars will follow the naming from here onwards, e.g. *Salmonella enterica* serovar Typhi; *S.* Typhi) ferments carbohydrates, to produce acid; however it produces little or no gas [4] . *S.* Typhi ferments glucose and manitol but does not ferment lactose, sucrose, salicin or adonitol. The O-nitrophenyl-3-D-galactopyranoside (ONPG) test is negative with respect to *S.* Typhi [5]. Some exceptional strains of *S.* Typhi do not ferment arabinose or dulcitol. Similar to other *Salmonella* species, *S.* Typhi decarboxylases the amino acids, lysine, ornithine and arginine, but cannot decarboxylate glutamic acid [1].

1.4 *Salmonella* serotyping

Members of the genus *Salmonellae* are distinguished from other enteric bacteria on the basis of their physiological characteristics. The *Salmonellae* are classified into different serotypes by the Kauffmann-White scheme [2]. The Kauffmann-White scheme (serotyping) is based on the immuno-reactivity of two main surface structures, the O antigen (somatic) and H antigen (flagella). The various *Salmonella* serotypes are identified by biochemical testing of these two antigenic structures. The O antigens and the H antigens are detected in independent agglutination assays using specific antisera that react with the group of related antigens or a single antigen.

Primary identification is based upon antibody agglutination of specific repeating units of the bacterial lipopolysaccharide (LPS). The LPS is assembled of between four and six sugars, depending on the O antigen [6]. They are hydrophilic and enable the bacteria to form stable, homogeneous suspensions in saline solution [1]. To date, over 60 different *Salmonella* O antigens had been recognized and are designated by numbers and are divided into O serogroups [1].

The H antigen, the filametous portion of the bacterial flagella, is made up of repeating protein subunits, called flagellin. The end region of flagellin is conserved and gives the filament its characteristic structure [6]. The antigenically variable portion of flagellin is the central region of the protein, which is exposed on the surface [6]. The majority of the genus *Salmonella* can express two different H antigens, which are encoded by two different genes, i.e. they are biphasic and the flagella are referred as Phase 1 and Phase 2. However, due to gene regulation only one gene can be expressed at a single time in an individual bacteria cell. Organisms such as *S.* Typhi express only one flagella type and is monophasic. In phase I (the specific phase), different antigens are designated by small letters, a to z. The phase 2 antigens (the group phase) first discovered were given Arabic numerals, but then, certain phase I antigens, especially e, n, x and z were also found to be present in the phase 2 of some strains [1]. Some *Salmonella*, for example, *S.* Typhi can express an additional antigen, known as the Vi capsule. Vi is thought to be vital for the pathogenesis of the organism and can also be detected by specific antisera and is therefore an additional identification test.

The combination of the O and the H antigen (and Vi) give the organisms a specific antigenic structure, of which each variant is called a serovar. Serotyping is essential for

laboratory identification of the *Salmonellae*, on the basis of testing there are currently over 2,500 different *Salmonella* serovars [4].

1.5 Nomenclature

Each individual serovar is given a specific name to identify the organism. Originally the organisms were named by medical microbiologists to reflect the disease they caused e.g. *S.* Typhi (as the organism causes typhoid fever) and *S.* Typhimurium (causing typhoid in mice). Latterly serovars were named after the location they were first discovered, e.g. *S.* Newport, *S.* Sendai and *S.* Stanleyville.

1.6 *Salmonella* disease in humans

The majority of the members of sub-species I of the genus *Salmonellae* are zoonotic and can survive in the gastrointestinal tract of their vertebrate hosts and in the environment [7]. Transmission generally occurs via the faecal – oral route, whereby a potential host eats or drinks contaminated food or water. The nature of the infection in humans is dependent on the serovar, the strain, the dose and the status of the host. The *Salmonellae* are generally associated with a self-limiting gastrointestinal infection in humans [7]. Ingested bacteria are consumed, usually in contaminated foodstuffs like dairy products and colonize the small intestine where they stimulate inflammation. *Salmonella* food poisoning causes stomach cramps, persistent diarrhea and vomiting and can last for several days [8]. It is seldom life threatening, but complications can occur in the elderly or the immunocompromised [9]. *Salmonella* food poisoning (gastroenteritis) can be caused by wide spectrum of *Salmonella* serovars and is a global problem [10-12].

Some members of the genus *Salmonella* are non - zoonotic and are host adapted. Host adapted means that they can only cause a specific disease syndrome in an individual host [13]. For example, *S.* Typhimurium causes a typhoid like disease in mice and a gastrointestinal infection in humans. The level of host specialization is further exaggerated by *Salmonella* that are host restricted, this means that the organism can only cause a disease in a specific host. *S.* Typhi is probably the best example of this as it causes typhoid fever in humans and higher primates but does not infect any other hosts; therefore, humans are the only known reservoir of infection [14].

1.7 Typhoid (enteric) fever

Typhoid fever is a severe systemic infection caused by the bacterium *S.* Typhi. The disease typhoid is classically characterized by a fever which is generally prolonged and is caused by bacteria entering the bloodstream [9]. A similar disease syndrome is caused by other human restricted *Salmonella,* serovars Paratyphi A, B and C, however, these organisms are less common than *S.* Typhi and are geographically isolated [15].

The term "typhoid fever" is interchangeable with the term "enteric fever", but enteric fever is the disease name for all human invasive *Salmonellae*, whilst typhoid is only associated with the bacterium *S.* Typhi. Enteric fever is seldom fatal in those patients whom are appropriately managed, but may result in fatality if untreated; the mortality is related to complications and it may be as high as 10% in untreated individuals [16] and varied from 4.8 % to 30.5% in children [17]. Due to improvements in sanitation, enteric fever no longer poses a serious health problem in developed countries. The main burden of enteric fever lies in developing and undeveloped countries where inadequate sanitation appears to aid the ongoing transmission of the organisms [18].

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1.7.1 Clinical features of typhoid

Symptoms of typhoid fever vary from a mild illness with low-grade fever, malaise and a dry cough to a more severe manifestation with abdominal discomfort and multiple complications [19]. The disease may be categorized into two groups on the basis of clinical features and severity, these are described as below:

Acute non - complicated typhoid fever is characterized by prolonged fever, disturbances of bowel function (diarrhea or constipation), headache, malaise and anorexia. A bronchial cough is a common symptom in the early stage of the illness. During the period of fever, 25% of patients show rose spots on the chest, abdomen and back [19].

About 10% of typhoid patients with acute typhoid fever may progress and develop serious complications. Approximately 10 – 20 % of patients with severe illness present with bloody stools, abdominal discomfort or intestinal perforation [19]. Other symptoms such as neurological complications, such as hallucinations occur, but are less reported [19].

The clinical features of other enteric fevers (associated with *S.* Paratyphi serovars), are historically considered milder than those associated with *S.* Typhi [15]. However, a prospective, descriptive study from Kathmandu, Nepal, found no difference in disease severity or duration in patients infected with *S.* Typhi or *S.* Paratyphi A [20]. It is believed that *S.* Paratyphi A has a shorter incubation period and can manifest with jaundice. Relapse may also be common in patients with *S.* Paratyphi A, which maybe occur in up to 8 % of patients, depending on location and the treatment [15].

1.7.2 Diagnosis

Since the symptoms of typhoid fever are often non - specific, the disease is often difficult to diagnose clinically. Owing to the locations where typhoid is endemic the disease syndrome may be confused with other illnesses where patients are febrile [16]. In general, typhoid should be considered in the differential diagnosis in those patients who have not been confirmed to have malaria, pneumonia, appendicitis or meningitis [21]. Whilst there are several methods of diagnosis which are commonly used, there is only one method which permits a guarantee that the patient has typhoid fever. The mainstay of enteric fever diagnostics relies on the isolation of *S.* Typhi (or other invasive *Salmonellae*) from the peripheral blood, faeces or bone marrow [21]. The sensitivity of blood culture decreases with the duration of illness and the use of antimicrobials prior to blood collection greatly reduces the isolation rate [21].

Bone marrow culture is a more sensitive procedure, but due to the invasive nature of the method it is not generally routinely performed [22]. Culture from stool is less sensitive than blood and bone marrow culture, owning to the transient nature of shedding the organism [9]. Isolation of the pathogen from faecal specimens is the only realistic method for identifying asymptomatic carriers, thus making identification of these people a considerable challenge.

There are several serological tests available for the diagnosis of typhoid fever, both in the form of laboratory assays and point-of-care diagnostic tests. The classical typhoid diagnostic test is the Widal test, which is used to identify antibodies against the O (Somatic) and the H (flagella) [23]. *S.* Typhi antigens which usually appear a week to 10 days after disease onset. Therefore, the Widal test utilizes paired serum samples from the acute and the convalescent phase of the infection. The Widal test method is

often a test of choice in many endemic locations yet is difficult to interpret due to false-positive and false-negative results [19,24]. A single sample method for the Widal test was assessed in Viet Nam; this study found that whilst this method did have some clinical use, the test is limited due to cross-reactivity of other bacterial antigens and the exposure rate in an endemic location [25].

Newer diagnostic tests have been developed to confirm typhoid fever quicker and may offer a higher degree of sensitivity and specificity. These tests are mainly based on the binding of specific antibodies to *S.* Typhi antigens and maybe more sensitive and specific than the Widal test [19]. However, the cross reactivity of bacterial antigens also hinders such newer generation tests, such as Typhidot and Tubex [19]. These assays were assessed in population-based typhoid surveillance studies in several countries and in all locations the sensitivity and specificity for Tubex and Typhidot was only around 70% and 80% respectively [26,27].

Molecular diagnostic approaches have also been introduced to improve typhoid fever diagnostics such as nested and real time PCR [21,28]. However, a molecular approach using PCR, despite its advance in methodology is relatively complicated and costly to perform, and is probably not a suitable routine use test in clinical microbiological laboratories where typhoid is endemic [9].

1.7.3 Epidemiology

It is estimated that typhoid caused 21.7 million new infections and 216,510 deaths in the year 2000 [29]. Humans are the only natural host and reservoir of *S.* Typhi and the main sources of infection are food and water contaminated with faeces containing the organism [19]. Little is known about direct transmission of the organisms, but disease is

associated with poor sanitation and it is suggested that *Salmonella* have the ability to survive for prolonged periods within the environment [30,31]. As the organism is transmitted in this maner, children, like with many other enteric pathogens are most at risk from the disease, due to increased contact and a potential lack of immunity [32,33].

Due to lack of sanitation, typhoid fever incidence in developing countries is significantly higher than that of industrialized countries and the incidence is highly dependent on the location [29]. In 2000, the incidence of typhoid fever in Asia was reported as the highest rate globally with 274 per 100,000 persons per year [29]. The areas of high incidence in Asia were in South Central Asia (622 / 100,000 / year) and South East Asia (110 / 100,000 / year). In other locations in Asia the incidence of typhoid fever was negligible. For example in Taiwan < 3 per 100,000 people per year were infected and 25 % of these cases originated from travel to other South East Asian countries [34]. On the African continent the incidence is approximately 50 cases per 100,000 people per year and the highest rate of infection is found in South Africa, with 233 cases per 100,000 people per year [15].

The incidence of typhoid fever continues at low levels in developed countries and reported cases are generally imported by travelers returning from areas which are endemic. Amongst 282 cases of reported typhoid cases in the United States from June 1, 1996 to May 1, 1997, the patient travel history could confirm disease in 81 % (229 / 282) of these [35]. Similarly, the incidence of typhoid fever in the United Kingdom and Australia is less than one case per 100,000 people per year [29,36].

Some simplistic steps may be employed to prevent the transmission of typhoid fever in endemic areas and in travelers to these locations. Specifically, attention should be made

to appropriate food handling procedures and hand washing with soap before preparing or eating food. In endemic areas other simple strategies such as avoiding consuming uncooked food, shellfish and taking ice in drinks may prevent infection [19].

1.7.4 Epidemiology of typhoid in Viet Nam

The nationwide incidence rate of typhoid fever in Viet Nam is approximately 15 per 100,000 people per year; with 56 % of cases occurring in children between the ages of 5 to 14 years [37]. The highest burden is isolated mainly to six provinces (Dong Thap, An Giang, Kien Giang, Can Tho, Vinh Long, Soc Trang) [38]; these provinces have incidence rates of greater than 100 / 100,000 people per year [37,38]. Like in other endemic areas the majority of the infections in the south of Viet Nam occur in locations which lack an adequate clean water supply [39]. These places have been shown to have water with high feacal contamination, a low level of general public hygiene and limited access to good sanitation [40].

Between 1993 and 1994, three serious outbreaks occurred in southern provinces of Viet Nam. These included Kien Giang (1993) with 2,459 cases, Soc Trang (1994) with 520 cases and Ho Chi Minh City (1994) with more than 400 defined cases [38]. The outbreak in Ho Chi Minh City was of particular importance due to the isolated organisms having a high rate of multidrug resistance [38].

Using pulsed-field gel electrophoresis, bacteriophage typing, plasmid typing, and antimicrobial susceptibilities, Connerton *et al.* demonstrated that independent outbreaks of multidrug resistant typhoid fever in southern Viet Nam were caused by a single bacterial strain [41]. However, different outbreaks were not derived from the clonal expansion of a single multidrug resistant *S.* Typhi strain. The clonal transmission of *S.*

Typhi may be a consequence of the inhabitant's habit of using river and rain water as a primary source of water for drinking and cooking [42]. During the period of high incidence in Viet Nam, 90% of the households in the Mekong Delta region were using "fish pond latrines", a latrine over a pond used to cultivate fish for farming. Surveillance also showed that the peak incidence of typhoid in Mekong Delta was at the beginning of the wet season in March and April, a period when the water table is high and the potential for drinking water to become mixed with effluent, from fields or other sources of faecal contamination may be higher [42].

1.7.5 Prevention of typhoid fever

In addition to improvements in sanitation and improvements in public health, typhoid is a vaccine preventable disease [19]. Vaccination for typhoid is cost effective and highly efficacious. The first parental vaccine (a killed whole cell vaccine) was introduced in 1896 and was shown to control the disease in Thailand. However, the vaccine produced strong side effects, presumably due to the LPS and the immunogenic nature of other bacterial proteins [19,43]. This live, killed vaccine caused local discomfort and swelling in between 25 – 50 % of all recipients, and is no longer recommended for routine use [9].

Two safe and effective vaccines are now commonly available and recommended by the WHO. Ty21a is a live attenuated vaccine, which is taken in three doses over a week [19]. This vaccine is used less than the Vi polysaccharide vaccine due to cold chain limitations and requires a strict adherence to the dosing schedule. There are other live attenuated typhoid vaccines in development, namely CVD909 and M01ZH09. CVD909 is an *aroC/aroD/htrA* mutant which was engineered to constitutively express the *S.* Typhi Vi antigen [44]. M01ZH09 is an *S.* Typhi strain also containing an *aroC* mutation

combined with an *ssaV* mutation which prevents expression of the SPI-2 Type Three Secretion System (TTSS) [45].

The most commonly used vaccine is a single dose vaccine containing purified Vi polysaccharide [43]. The vaccine is suitable used for adults and children above two years of age and has no serious side effects. Production of Vi vaccines in non - industrialized countries such as Viet Nam has greatly reduced the cost of this prophylaxis and the Vietnamese ministr y of health has an ongoing Vi vaccination programme [46]. Studies conducted in Nepal and South Africa have demonstrated that the Vi vaccine provided two years protection and had a three year cumulative efficacy of 55 % [47,48]. A new modified Vi conjugate vaccine bound to non - toxic recombinant fragment of *Pseudomonas aeruginosa* exotoxin A (rEPA) has enhanced immunogenicity in adults and in children between 5 - 14 years of age, and provided induced booster responses in children aged between 2 - 4 years [49]. The Vi conjugate vaccines that are currently under development are widely considered as the future in terms of controlling typhoid fever [39]. Yet Vi based vaccines do not protect against *S.* Paratyphi A or B, since these strains do not express the Vi polysaccharide. Therefore, attenuated vaccines which may confer cross-protection against other invasive *Salmonella* [50] should also be considered for typhoid prevention in countries with high rate of paratyphoid fever [51].

1.8 *Salmonella* Typhi pathogenicity

Like other *Salmonellae*, *S.* Typhi has specific virulence machinery that permits the organism to live a pathogenic lifestyle [52,53]. The cornerstone of *Salmonella* pathogenicity are *Salmonella* Pathogenicity Islands (SPI's), horizontally acquired fragments of DNA which are inserted into the genome of the organism and contain a

multitude of virulence associated genes [54]. Overall, the mechanisms of *S.* Typhi pathogenicity are complex and rely on a number of coordinated events to permit invasion of the intestinal mucosa and intracellular survival. However, it is these gene islands and the ability to invade the gut mucosa and then survive in deeper tissues which distinguish these organisms from other non - pathogenic enteric bacteria [55].

Whilst the overall mechanisms of pathogenicity cannot be explained simply by two individual gene islands, SPI-1 and SPI-2 are vital for invasion and intracellular survival and are, therefore, the most well described and the most important pathogenicity islands in the genus *Salmonella* [56,57].

SPI-1 is an approximately 40 Kb gene island encoding a type three secretion system and multiple effector proteins which are essential for the organisms to invade the intestinal mucosal surface [58]. Type three secretion systems are biological syringes carried by some pathogenic organisms which permit the secretion of proteins (effectors) from the bacterial cell into the host cell. The secreted proteins can influence host cell function and facilitate bacterial invasion. The type three secretion system carried on SPI-1 is involved in the early stages of infection and acts at the Peyers patches (the location of invasion of *Salmonella*) to facilitate invasion of the cell [59].

Similar to SPI-1, SPI-2 is an approximately 40 Kb gene island encoding a type three secretion system and multiple effector proteins [53]. However, the type three secretion system encoded by SPI-2 plays an entirely different function to SPI-1, yet is also essential for the pathogenesis of the *Salmonellae.* SPI-2 is involved in the survival and persistence of infection in *Salmonella* once the organism has invaded the mucosal surface. The type three secretion system and its secreted effectors are vital for systemic

infection in mice and permit the organism to survive intracellularly inside macrophages [60].

The pathogenesis of typhoid disease in man has received only limited attention, due to the host restricted nature of the organism and complexity of studying infection with a human host. The incubation period for *S.* Typhi in a particular individual depends on the quantity of inoculum and usually ranges from 3 days to more than 60 days [19]. Experimentation using surrogate hosts and *S. enterica* serotypes (e.g. *S.* Typhimurium) suggest that tissue invasion occurs predominantly through M cells on Peyers patches in the terminal ileum [61]. Tissue invasion drives a potentially close encounter with the immune system. However, *S.* Typhi is an immuno-modulatory pathogen which goes to great lengths to avoid detection by the immune host defenses. *S.* Typhi multiplies in mononuclear phagocytic cells before being released into the bloodstream and spreads throughout the body. As a result of this silent primary bacteraemia, the pathogen reaches one of several organs, such as spleen, liver and bone marrow (probably within 24 hours after ingestion), through the reticulo-endothelial system [62]. The infection eventually localizes to the gall bladder where the internal transmission cycle is completed as *S.* Typhi organisms are shed in bile. Chronic carriers are a major public health problem as *S.* Typhi persists within the gallbladder and the bacteria can be shed in faeces for years which subsequently may contaminate food or water and initiate transmission to other people [63]. Whilst these mechanisms have never been proven for typhoid, it is clear that by utilizing SPI-1, SPI-2 and other virulence factors that *S.* Typhi has predominantly forsaken the luminal lifestyle of most enteric bacteria.

In contrast with *S.* Typhimurium, which can (but not routinely) disseminate from the intestine and cause bacteremia with a rapid onset of symptom which progresses to

sepsis shock, *S.* Typhi has a gradual onset of symptoms and does not rapidly progress to hypotension [64]. A recent study conducted on *S.* Typhimurium has shown that expression of the Vi capsule but not expression of the TviA regulatory protein (a protein encoded by a gene in the ViaB locus which regulate the expression of Vi capsule) reduced tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) in murine bone marrow derived macrophages. The same study also found that the Vi capsule prevented both *in vitro* and *in vivo* recognition by TLR-4 and the expression of interleukin 8 (IL-8) and FliC was modulated by the TviA regulatory protein but not by the Vi capsular antigen [65]. These data suggest a role for flagella gene regulation in the process of human adaption that is seen in *S.* Typhi.

1.9 The evolution and genomics of *Salmonella* Typhi

In 2001, the genome sequence of an *S.* Typhi strains from southern Viet Nam was sequenced and it represented the first genome of the *Salmonellae* to be sequenced in its entirety. The organism was named CT18 and was found to encode over 4,000 genes and contain multiple insertions and deletions when compared to *E. coli* K12, these insertions ranged from individual genes to large islands. The genome sequence additionally identified over two hundred pseudogenes (inactivated genes) [66], demonstrating that the organism is in an ongoing state of evolution. Gene acquisition and somewhat more surprisingly gene degradation appear to have generated the host restricted pathogen we find circulating in human populations today [67]. The CT18 genome sequence also contained two plasmids, named pHCM1 and pHCM2. pHCM1 was a 218,160bp *incHI* plasmid that conferred multiple antimicrobial resistance. Whilst pHCM2 was a 106,516 bp plasmid and was believed to phenotypically cryptic but demonstrated some sequence homology with the *Yersinia* pestis virulence-associated plasmid, pMT1 [66].

It is understood from comparing DNA sequences from related bacteria that *S.* Typhi has been in existence for approximately 50,000 years since evolving from its last common ancestor [68]. Using single nucleotide polymorphisms (SNPs) to identify genetic variation demonstrated that evolution from the last common ancestor probably arose on the African continent [69]. Whilst sequence diversity within the genome may be limited, the organisms are known to exhibit some genetic re-arrangements and some areas of the genome which may demonstrate diversity due to the acquisition of gene clusters, this is described as genome plasticity [67,69].

Defining the circulating population is particularly challenging for studies of *S.* Typhi, as this organism is monophyletic and sequence diversity is limited [70]. This was demonstrated by the sequencing of 199 gene fragments from a global collection of 105 *S.* Typhi isolates detected only 82 SNPs [71] . This study described the molecular epidemiology of various *S.* Typhi genotypes and generated a population structure on the basis of the evolution of nucleotide variation. Fifty-nine haplotypes of *S.* Typhi were found to circulating globally, of which one haplotype, named H58 was found to be the dominant genotype circulating in southern Asia in recent years. There is a strong correlation of the H58 haplotype with the presence of mutations in the topoisomerase genes related to nalidixic acid resistance [71]; this is presumed to be related to antimicrobial selective pressure on the population [71]. However, H58 is not a unique genotype related to a nalidixic acid resistant phenotype, but represents a clonal population expansion. Often local populations of *S.* Typhi consist of a mixture of nalidixic acid sensitive and resistant organisms of the same haplotype [71,72].

The formation of a rooted phylogenetic tree based upon these SNPs permitted greater understanding of the global population of *S.* Typhi and provides a potential system for

tracking the pathogen in an endemic setting. By assaying the varying SNP loci Baker *et al.* were able to identify several genetically distinct *S.* Typhi haplotypes circulating in an urban area of Jakarta, Indonesia and demonstrate the clonal nature of antigenic variation within the local population [73].

Only limited studies have been performed on the *S.* Typhi strains circulating in Viet Nam. Roumagnac *et al.* found four haplotypes (H1, H50, H58 and H63) of *S.* Typhi strains in Viet Nam. Of these haplotypes, two (H1 and H50) have been persisted in the population for at least 37 years until 2004. As in other locations the H58 haplotype with mutations in topoisomerase genes is the predominant strain currently in southern Viet Nam [71]. A potential hypothesis explaining the clonal expansion of H58 is the use and selective pressure of antimicrobials in Viet Nam. Throughout the 1990's this haplotype became dominant and spread to other locations, however, this is unproven and spread may be unrelated to use of fluoroquinolones.

More recently, high-throughput sequencing technologies were used to determine the genomes of 19 *S.* Typhi isolates that were selected to maximize diversity according to available variation data. This study revealed nearly 2,000 additional SNPs within the *S.* Typhi population, providing additional loci for SNP typing of clinical isolates. Importantly, the study detected relatively few examples of other forms of genetic variation such as insertions and deletions (fewer than 30 in total), which could potentially be used as phylogenetically informative markers for studying *S.* Typhi populations [69].

1.10 Antimicrobial agents and antimicrobial resistance mechanisms

The management of typhoid fever is relatively straightforward, as all typhoid patients are treated with antimicrobials and many patients can be treated effectively as outpatients. For those that are treated as inpatients, good nursing care, adequate nutrition, attention to fluid and electrolyte balance and prompt treatment of complicated cases are vital to prevent mortality. Appropriate antimicrobial therapy effectively reduces the illness's morbidity, mortality and complications. The deployment of appropriate antimicrobials is essential for the treatment of this infection [74].

Typhoid fever was first treated by chloramphenicol, ampicillin and trimethoprim-sulfamethosazole in the 1960's. The emergence of multidrug resistant *S.* Typhi strains which resisted to chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole urged the need of more effective antimicrobials. Fluoroquinolones have been used as the drugs of choices for typhoid treatment with third generation cephalosporins and azithromycin as alternatives [74].

1.10.1 First line antimicrobials

Chloramphenicol was introduced in 1948 and shows remarkable effectiveness for enteric fever treatment worldwide [75]. Chloramphenicol binds to the 50s bacterial ribosomal subunit and inhibits bacterial growth by inhibiting protein synthesis. In the 1960s, *S.* Typhi strains with plasmid-mediated resistance to chloramphenicol appeared and later these strains became widespread in many endemic countries in America and South East Asia, urging the need for alternative agents [75].

Amoxicillin and trimethoprim - sulfamethoxazole became the mainstay of typhoid treatment in 1970's [76]. Amoxicillin disrupts the synthesis of cell wall mucopeptides

during active multiplication, resulting in bactericidal activity against susceptible bacteria. Trimethoprim - sulfamethoxazole inhibits bacterial growth by inhibiting dihydrofolic acid synthesis.

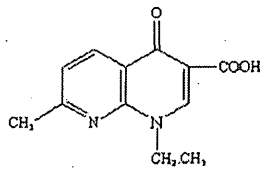
Chloramphenicol, amoxicillin and co-trimoxazole are appropriate treatments for typhoid fever in areas where the bacteria are still susceptible to these drugs. The advantages of these drugs are that they inexpensive, widely available and rarely associated with side effects. These antimicrobials have a high cure rate (95 %), a low relapse rate (1 - 7 %) and a rate of convalescent excretion of between 2 - 10 % [9]. According to the WHO, 1 - 5 % of untreated typhoid patients progress to become chronic carriers, this is reduced by treatment with first line antimicrobials [19].

Cefotaxime and ceftriaxone, which are third generation cephalosporins, arrest bacterial cell wall synthesis and hence inhibit bacterial growth. These antimicrobials have high power *in vitro* against *S.* Typhi and other *Salmonellae* and have acceptable efficacy in the treatment of typhoid fever [75].

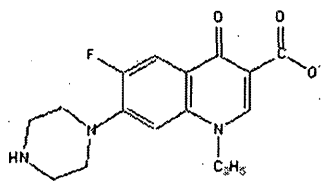
1.10.2 Fluoroquinolones: the structures, mode of action and resistance mechanisms

Studies have shown that fluoroquinolones are safe and effective in fluoroquinolone-susceptible, uncomplicated typhoid fever [9]. Fluoroquinolones and 4-quinolones are derivatives of nalidixic acid, which was the first analogue released for treatment of urinary tract infections in 1962, this group was the first man-made class of antimicrobials [77].

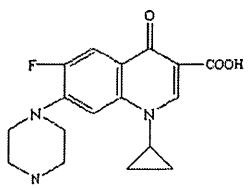
Nalidixic acid shows a narrow antibacterial spectrum; it has a short half-life but high protein binding efficiency [78]. Norfloxacin, the first generation of fluoroquinolone, was introduced with a piperazine at position C7 (with respect to nalidixic acid), it demonstrates broader Gram negative activity, with a longer half life but less protein binding capabilities when compared to nalidixic acid [78]. The second generation of fluoroquinolone was created with the addition of fluorine at position C6, thus increasing the activity against the target (DNA gyrase) and having increased penetration into the bacterial cell [78]. The three-ringed benzoxazyne fluoroquinolones (ofloxacin and perfloxacin) are connected at positions C8 and N1 with a bridge which contains an oxygen molecule and two carbon atoms [78]. While ofloxacin contains stereoisomers, R- and S-enatiomers, levofloxacin has only S-enantiomer that is 10- to 50- fold more potent than the R-enantiomer. The cyclopropyl moiety group attached at position N1 of ciprofloxacin enhances bactericidal potency compared to the older fluoroquinolone generations [78], this was proved in animal model of infection [79]. Amongst the third generation of fluoroquinolones, gatifloxacin with a methoxyl group at C8 has greater potency against Gram-positive bacteria by targeting both topoisomerase II and IV and also has good activity against anaerobic bacteria. Trovafloxacin, moxifloxacin and gemifloxacin are fourth generation fluoroquinolones and have potent activity against anaerobes and increase activity against pneumococci [78,80-82].



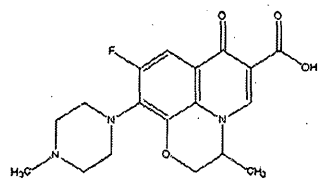
Naidixic acid



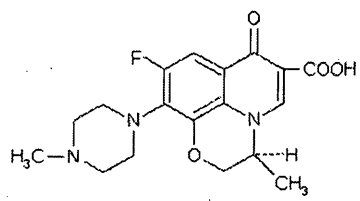
Norfloxacin



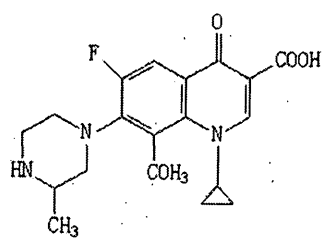
Ciprofoxacin



Ofloxacin



Levofloxacin



Gatifloxacin

Figure 1.1 (Fluoro)quinolones representatives structures

See text for description of structure.

1.10.2.1 Gyrase and topoisomerase IV as therapeutic targets

The quinolones and fluoroquinolones target the topoisomerase enzymes in the bacterial cell [83]. Topoisomerases are essential enzymes in DNA in replication, transcription and other cellular functions in bacteria [84]. Topoisomerases are categorized into 2 groups: type I and type II [84]. Type I enzymes rapidly break the DNA strands individually. Type II enzymes transiently break the pair of strands in the DNA double helix in concert by a dimeric enzyme molecule [84]. The two types of topoisomerases can be further divided into four subfamilies: IA, IB, IIA and IIB. Members of the same subfamily are structurally and mechanistically similar, whereas those of different subfamilies have distinct properties [85].

The topoisomerases are enzymes that maintain genomic integrity during replication by forming a covalent linkage between the active site tyrosine and the newly generated DNA termini via phosphotyrosyl bonds [83]. The covalent topoisomerase - DNA cleavage complexes are fleeting catalytic intermediates, but these breakages are of potentially great harm to the genome integrity [83]. Shortly after the discovery of these enzymes, the DNA gyrase and topoisomerase IV (topo IV) proteins were recognized as targets for antimicrobials.

1.10.2.2 Structures and functions of gyrase and topoisomerase IV

DNA gyrase and topo IV are type IIA topoisomerases, these enzymes are present in all bacteria [83,85]. Although, gyrase and topo IV only share approximately 40% amino acid sequence homology [86], they play fundamental but distinct roles in the bacterial cell. DNA gyrase is involved primarily in supporting nascent chain elongation during replication of the chromosome, whilst topo IV separates the topologically linked daughter chromosome during the terminal stages of DNA replication [83].

DNA gyrase is unique among type IIA enzymes in that it can introduce negative superhelical turns to DNA. Additionally, DNA gyrase can play a role during chromosome decatenation [83]. DNA gyrase requires ATP as a cofactor for activity [87]. The protein is a tetramer with two A and two B subunits which are encoded by *gyrA* and *gyrB* gene respectively [87]. The *gyrA* gene maps at 50.3 minutes on the *E. coli* chromosome and encode a 97 kDa protein (875 amino acids), whereas *gyrB* maps to 83.5 minutes and encodes a 89.9 kDa protein (804 amino acids). Topoisomerase IV is encoded by the *parC* and *parE* genes. The *parC* and *parE* genes map to 68.1 minutes and encode an 83.7 kDa protein and map to 68.3 minutes and encode 70.2 kDa protein

respectively. The ParC and ParE proteins are highly homologous with the GyrA and GyrB proteins.

The GyrA and ParC proteins are the subunits responsible for DNA binding at the cleavage and re-ligation reaction. They execute cleavage and re-ligation processes by forming a covalent phosphotyrosine bond between an active site tyrosine and the DNA phosphate backbone. The tyrosine active site is located at the amino acid residue 122 in GyrA and 120 in ParC. Both gyrase and topo IV cleave DNA in a site-specific manner, but site recognition is distinct between these two enzymes [83].

DNA gyrase was first recognized by its unique ability to introduce negative supercoils into DNA, which is a requirement for the negatively supercoiled DNA template for initiation of the replication bubble. DNA gyrase is wrapped around the enzyme in a positive supercoil. Subsequently, one segment of the duplex DNA is passed through another via DNA breakage and rejoining [83]. Topo IV also catalyzes the breakage and rejoining of DNA strands but does not wrap DNA during functional activity [83].

1.10.2.3 Mechanisms of quinolone action

As mentioned above, DNA gyrase is the primary target for the quinolones. The most potent group for activity of the quinolones is the cyclopropyl group at position N1 in ciprofloxacin [88]. At position C5, substitutions may increase activity due to enhanced permeability rather than to increase potency against the DNA gyrase [89]. Whilst the addition of a fluorine at C6 substantially improves potency, substitutions at C7 and C8 alters potency against DNA gyrase [90,91]. Among various modes of quinolones binding to DNA, the interaction with single stranded DNA, mediated by Mg^{+} correlates strongly with drug potency [92]. Quinolones bind to the DNA phosphodiester backbone

via a Mg^{+} bridge, which is coordinated by the C-3 and C-4 groups and requires guanine specific contact at the 4-base region downstream of the gyrase cleavage site. T he presence of guanines is important for higher-affinity interaction. In other words, the greater the affinity for single stranded DNA, the greater the potency of the quinolones [92].

1.10.2.4 Bactericidal activity of fluoroquinlones

Norfloxacin rapidly kills *E. coli* within one or two hours of exposure, then slows in activity on reaching a killing plateau [93]. The sub-population of surviving cells represents the persistant proportion of the population. Cells are killed by forming bacterial filaments, swelling (losing membrane integrity) and lysis [94]. The rate of bacterial killing by exposure to quinolones increases proportionally with drug concentration and eventually decreases at higher drug concentrations (30 to 60 times the minimal inhibitory drug concentration). A decrease in activity at higher concentrations is called the paradoxical bactericidal effect [95]. Therapeutically, the bactericidal activity of quinolones is decreased by chloramphenicol, rifampin, nutrient starvation and other treatments that also inhibit protein synthesis [95].

1.11 Molecular mechanisms of antimicrobial resistance

The resistance of bacteria to antimicrobials can occur either due to mechanisms driven by the functionality carried on the core genome (intrinsic) or be acquired from additional genetic sources (plasmids or bacteriophages). Acquired antimicrobial resistance genes are generally carried on plasmids, integrated into transposons or integrons and often can be transferred naturally from donor to recipient cells by the three mechanisms of DNA transfer, namely transformation, transduction or conjugation [96].

In principle, bacteria have three commonly described systems of initiating antimicrobial resistance [97]. The first process involves enzyme secretion from the bacterial cell. Organisms can produce enzymes that may degrade, destroy or alter the chemical agent. These enzymes break antimicrobials into small molecules that have little or no bactericidal effect on the bacterial cells. For example, those bacteria that are resistant to penicillins and cephalosporins can produce β-lactamase enzymes that break down these chemicals and prevent activity. In addition, other secreted enzymes can modify some antimicrobials. These modifying enzymes convert an activated antimicrobial into an inactivated variant. An enzyme produced by *Streptomyces spectabilis* which can acetylates the 2'-amino group of the hexose ring of gentamicin is an example of the modifying enzymes initiating resistance [98]. Limiting antimicrobial accumulation inside the bacterial cell is the second common resistance mechanism. Some membrane proteins of bacteria can be under-expressed by the bacterial cell to protect cells from chemicals entering the cell. Additionally, active efflux-pumps produced by protein over expression may additionally limit the bactericidal activity of the antimicrobial by actively ejecting the chemical agent from within the cell. Active antimicrobial efflux mediates resistance to tetracylines, chloramphenicol and the fluoroquinolones. The third resistance mechanism in bacteria is the ability of the organism to modify the target of the antimicrobial. For example, bacteria can modify ribosomes, metabolic enzymes or proteins involved in DNA replication and cell wall synthesis. Some of these three mechanisms can occur in tandem and often multiple mechanisms are required for a substantial increase in resistance levels.

Generally, many enteric bacteria are highly adaptable when placed under strong selective pressure. Antimicrobial resistant organisms can be positively selected on the basis of the antimicrobial (and other chemicals) they come in close proximity to.

Whether the organism produces a specific enzyme to destroy or alter antimicrobials, prevents drug transport into the cell or regulates the expression of a specific protein, all these mechanisms play a major role in bacteria survival, evolution and adaptation.

1.12 Fluoroquinolone resistance

Mechanisms of quinolones resistance can occur by all of the three main mechanisms of resistance as illustrated in Figure 1.1 [99]. The commonest mechanisms are mutations in the target site (generally the *gyrA* gene), efflux pumps and reduced permiability to chemicals, however more recently, fluoroquinolone resistance mechanisms related to plasmid and antimicrobial - modifying enzymes have been demonstrated [100].

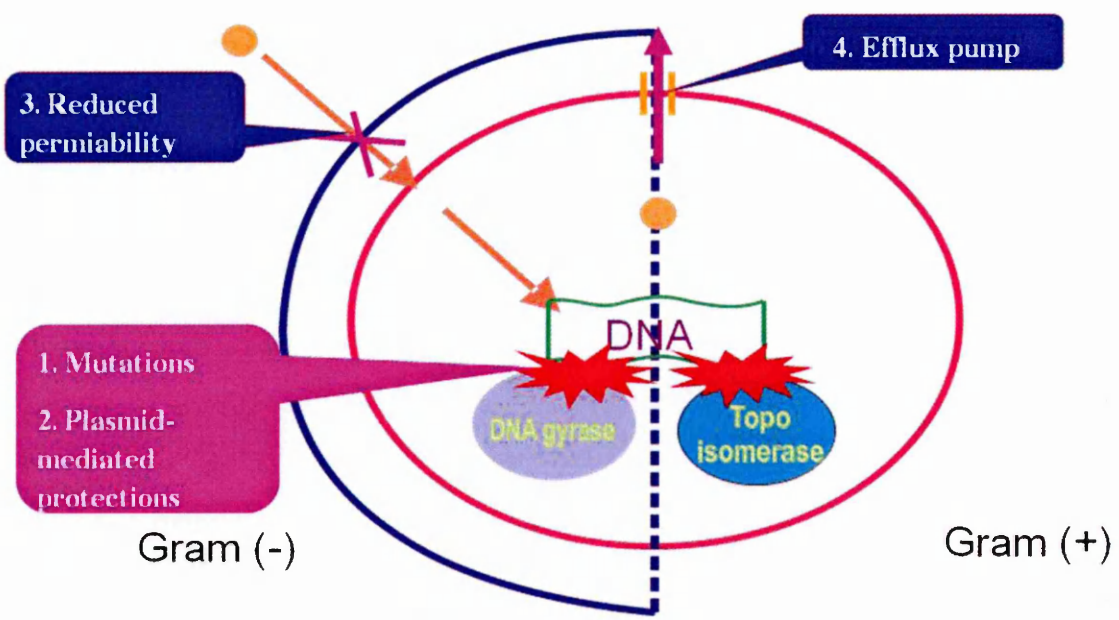


Figure 1.2 The three mechanisms of fluoroquinolone resistance

A schematic diagram of the three mechanisms of fluoroquinolone resistance was modified from Bambeke et *al.*[82]. These are (1) mutations in the target genes, (2) plasmid mediated resistance enzymes and reduced antimicrobial accumulation by (3) reduced permeability and (4) efflux pump enhancement.

1.12.1 Alterations in the gyrase and topoisomerase IV target enzymes

Mutations in the *gyrA* gene account for first step fluoroquinolone resistant mutants whilst mutations in the *parC* gene are second step fluoroquinolone resistant mutants [101]. Resistance mutations can also occur in the *gyrB* gene, often to intermediate [102] and sometimes to low quinolone concentrations [103]. The most common mutations causing fluoroquinolone resistance in *S.* Typhi occur at positions 83 and 87 in *gyrA* gene and at position 80 in the *parC* gene [104], whilst no mutations in *gyrB* gene or the *parE* gene have yet been detected in the *S.* Typhi population.

1.12.2 Alteration in drug permeability

To reach their targets in the cell cytoplasm, fluoroquinolones must cross the outer and cytoplasmic membrane. Depending on their hydrophobicity, fluoroquinolones pass through the outer membrane either directly through the lipid bilayers (nalidixic acid) or through water filled protein pores, the porins (ofloxacin, ciprofloxacin) [99,105]. Resistance due to reduced drug accumulation involves at least one or two different mechanisms. The first mechanism is an energy - independent passive mechanism which is based on structural alterations or reduced expression of porin proteins responsible for quinolone passage through the outer membrane [106]. The second mechanism requires energy and involves active efflux of the quinolones [107,108]. Efflux as a mechanism of antimicrobial resistance was originally described for tetracyclines [109] and is also described for fluoroquinolones. This resistance mechanism is related to mutations in the endogenous ArcAB - TolC mediated membrane efflux pump [110]. Thus, reduced porin channels in combination with enhanced efflux capabilities of AcrAB complete the resistance pathway from cytoplasm or inner membrane to the exterior of the cell [111].

1.13.1 Plasmid mediated quinolone resistance

Plasmid mediated resistance to quinolones was first identified in *Shigella spp.* and was considered rare [112]. However, since identification, plasmid mediated quinolone resistance was confirmed in *Klebsiella pneumoniae* in 1998 and is now common in other enteric bacteria [113]. The gene family responsible for plasmid mediated quinolone resistance is the *qnr* family. These genes encode the Qnr proteins, which have 218 amino acid residues and belong to the pentapeptide repeat family [114]. The Qnr proteins share homology with another pentapeptide, McbG, which protects the DNA gyrase from microbin B17 action. The Qnr proteins probably protect the DNA gyrase from inhibitory effect of the fluoroquinolones [114]. Hypothetically, the formation of a Qnr-gyrase complex occurs before the formation of cleavage complex. Another transferable gene responsible for fluoroquinolone resistance is the *aac (6') - Ib* gene. The *aac (6') - Ib* gene encodes an aminoglycoside acetyltransferase that is capable of acetylating and thus reducing the activity of certain fluoroquinolones. This gene solely does not stimulate a high level of resistance; however, when combined with other plasmid encoded resistance mechanisms (such as the *qnr* genes) it leads to a higher resistance level.

S. Typhi strains with reduced susceptibility to fluoroquinolone are now common in Asia and can be identified on microbial culture by resistance to nalidixic acid. Although fluoroquinolones are considered to be an effective antimicrobial with rapid therapeutic response, fluoroquinolone resistance has been reported in typhoid endemic regions, including India and Pakistan [115].

1.13 Determination of antimicrobial resistance

1.13.1 Measurement of resistance phenotype

Antimicrobial resistance is evaluated via measurement of resistance and killing capabilities of bacteria *in vitro*. For determination of the level of susceptibility or resistance to quinolones, disk susceptibility is efficiently used for screening of clinical isolates. Agar, broth dilutions and E - test for the determination of MICs are straightforward methods used in clinical microbiological laboratories. Such methods have also been used for distinguishing quinolone sensitive and quinolone resistant mutants [111].

The determination of bactericidal activity of quinolones is performed by measurement of colony - forming units (CFU) by dilution of cells and plating on nutrient agar media. Comparing killing effects of strains by chemicals (time - kill analysis) is the most effective method to measure bactericidal activity. The time - kill technique correlates with therapy and cure rate in patients and is a reliable means of tolerance determination and it useful for identifying any synergy or antagonism between two antimicrobial agents [116].

Bactericidal activity can also be determined by broth and agar dilution methodology. The agar dilution method immobilizes inoculums of bacteria in an agar - gel matrix and depends upon inactivation of the antimicrobial agent in order to determine regrowth of viable organisms after a defined period of incubation. The broth dilution method is performed by a standard broth dilution technique, as used for MICs. However, in the later stages of the experiment, sampling of dilutions with no visible growth is performed with a calibrated device to determine the concentration at which 99.9 % of the final inoculum is killed [116].

1.13.2 Determination of resistance mechanism

Determination of resistance caused by alterations in DNA gyrase can be accomplished in several ways. Identification of mutations conferring resistance is limited to laboratories which have the capabilities to perform molecular methods. Association of quinolone resistance to genetic loci linked to gyrase encoded gene may be accomplished by conjugation and transduction methods. It is also possible to introduce plasmid clones containing *gyrA* or *gyrB* genes from susceptible wild type strains into resistant strains or vice versa by transformation [117,118]. The cloning of resistance genes is a more definitive method and may also be accomplished by direct selection for quinolone resistance, using strains with altered chromosomal *gyr* genes [119]. Direct demonstration of enzyme activities can be determined by purification of the bacterial DNA gyrase. Assays of DNA gyrase activities and their inhibition by quinolones have been performed using purified DNA gyrase. An indirect method has been used to identify the presence of an altered DNA gyrase in quinolone resistant mutants. This method involves a comparison of the concentration of quinolone inhibiting ATP - dependent replicative DNA synthesis in treated mutant and wild type cells [95].

The determination of resistance caused by altered permeation can be identified by characterization of outer membrane proteins or measurement of quinolone accumulation. A reduction of outer membrane proteins can be checked by the use of lytic bacteriophages that attach specifically to proteins on the bacterial cell surface. The commonest method used for this is the 'spot test' in which bacteriophage exhibit a failure to initiate lysis or show a decreased efficiency of plaque formation with mutants when there are substantial decreases in appropriate respective porin.

1.14 Aims of study

The aims of this study are to describe and then further investigate the resistance patterns of *S.* Typhi organisms isolated in Viet Nam and in other localities in Asia. Furthermore, here I determine the common resistance mechanisms of *S.* Typhi to fluoroquinolones in Asia and the effect of these mechanisms on the bacterial population. The specific aims of this work are as follows.

1. To describe the antimicrobial susceptibility patterns of *S.* Typhi in Asia

2. To investigate the molecular mechanisms of nalidixic acid resistance and reduced susceptibility to fluoroquinolone in *S.* Typhi isolates and to evaluate the effect different mutations have on fluoroquinolone susceptibility and MIC.

3. To reconstruct the *gyrA* and *parC* mutations in *S.* Typhi BRD 948 (an attenuated *S.* Typhi strain) and characterize the effect on phenotype. Phenotypic screening with respect to MIC testing, time - kill analysis and biological costs experiments.

4. To genotype fluoroquinolone resistant and sensitive *S.* Typhi isolates using MLPA (Multiplex Ligation dependent Probe Amplification) in comparison to the GoldenGate SNP typing technique. This will permit the development of a simplified typing scheme that can be applied universally to *S.* Typhi populations and can be used in resource poor settings.

2. Materials and Methods

2.1 Bacterial strain collection

2.1.1 Clinical isolates

The *S.* Typhi strains used in this study were isolated from clinical samples from research programmes conducted in Viet Nam from 1993 to 2005 and from seven other Asian countries from 2002 to 2006 (Table 2.1).

Table 2.1 The *S.* Typhi collection from Viet Nam and other Asian countries used for analysis in the study

No	Country	Year	Number of strains
1	Bangladesh	2003	40
2	China	2002	21
3	India_2003	2003	23
4	India_2006	2006	259
5	Indonesia	2002	17
6	Pakistan	2002-03	34
7	Laos, Mahosot Hospital	2002-03	50
8	Nepal- Patan hospital	2003	149
9	Central Viet Nam	2002-04	47
10	Southern Viet Nam	1993-2005	1388
	Total		**2028**

2.1.1.1 *S.* Typhi isolated in southern Viet Nam from 1993 to 2005

One thousand three hundred and eighty eight *S.* Typhi strains were isolated consecutively from patients with uncomplicated typhoid fever during prospective hospital-based clinical studies between 1993 and 2005 conducted at four hospitals located in southern Viet Nam, namely Dong Thap Provincial Hospital, Dong Nai Pediatric Hospital, An Giang Provincial Hospital and the Hospital for Tropical Diseases, Ho Chi Minh City. Results from these studies have been reported in several publications [120-127].

2.1.1.2 *S.* Typhi isolates from eight Asian countries from 2002 to 2006

The Asian isolates used in this study were collected in a several locations from a number of investigations. These locations and strains are described in turn. One hundred and forty-nine *S.* Typhi isolates were collected between March and April 2003, during a hospital-based descriptive study at Patan Hospital, Kathmandu, Nepal [20]. Fifty isolates were collected consecutively during a clinical trial in 2002 and 2003 at the Wellcome Trust-Mahosot Hospital, in the Lao People's Democratic Republic [128]. One hundred and eighty-two *S.* Typhi were collected as part of population-based prospective surveillance studies conducted by multiple teams in collaboration with the International Vaccine Institute (IVI), Seoul, South Korea [129]. These surveillance sites included townships (China, Viet Nam), specific temporary urban settlements (Bangladesh, Pakistan, and India) and an impoverished urban sub-district (Indonesia). In the collection contributed by the IVI, forty isolates were collected between February and December 2003 in a temporary urban settlement in Dhaka, Bangladesh [130]. Twenty one isolates were collected during 2002 in Hechi city, Guang Xi, China; 23 strains were collected from May to July 2003 in an urban area in Kolkata, West Bengal, India along with 259 strains collected across India in 2006; 17 isolates were collected from July to September 2002 in North Jakarta; Indonesia; 34 strains were isolated between January 2002 and March 2003 in one slum area in Karachi, Pakistan and 47 isolates were collected between July 2002 and September 2004 in Hue city, central Viet Nam.

All *S.* Typhi isolates were collected from febrile patients during the indicated period and from geographically contiguous areas. The isolates were believed to be an accurate representative of the strains circulating in the population they came from.

2.1.2 Non - clinical isolates

2.1.2.1 *S.* Typhi BRD 948

S. Typhi BRD 948 was a gift from the Sanger Institute in Cambridge, United Kingdom. *S.* Typhi BRD 948 is a laboratory mutant strain which is certified for use at containment level 2 and has been described and used extensively [131]. *S.* Typhi BRD 948, is a derivative of *S.* Typhi Ty2 with deletions in the *aroC, aroD* and *htrA* genes. This *S.* Typhi strain was used as the parent *S.* Typhi in mutation reconstruction and the *in vitro* selection of mutant experiments. The *aroC* and *aroD* genes encode enzymes of the aromatic amino acid pathway, thereby rendering the bacteria auxotrophic for para-aminobenzoic acid (PABA) and dihydroxybenzoate (DHB). The *htrA* genes encodes a heat shock protein (DegP), when mutated in *Salmonella,* the mutant is less virulent because of impaired ability to survive and replicate in host tissues [132,133]. The *S.* Typhi BRD 948 strain has been shown to be safe for genetic manipulation and so overcomes any potential issues associated with the introduction of antimicrobial resistance genes into wild type strains [118].

2.1.2.2 The *Escherichia* Coli *(E. coli)* strains

E. coli CC118λ*pir* and *E.* coli SY327λ*pir* were used as hosts for the suicide vector pJCB12 and its derivatives. These strains, which contain a functional *pir* gene in the chromosome, are essential for the replication of the recombinant plasmids generated in the mutagenesis experiments.

2.1.3 The suicide vector pJCB 12

Constructed mutants were made using the suicide vector pJCB12 (Figure 2.1). This suicide vector is a derivative of the vector pDM4, and has more than 50 % of the nonfunctional DNA removed. The vector pJCB12 retains the *ori*R6K origin, *mob*RP4

mobilization origin, multiple cloning sites, and the *cat* and *sacB* genes. In particular, the *sacB* gene en codes levansucrase, this chemical converts sucros e to levan which is harmful to the bacteria [134]. Thus, bacteria containing this suicide plasmid cannot survive on media containing sucrose. The *cat* (chrolramphenicol acetyl transferase) gene which encodes chloramphenicol acetyl transferase enzyme inactivates chloramphenicol by formation of mono – and diacetylated derivatives [134]. Therefore, bacteria containing functioning *cat* gene are resistant to and can be selected on media containing chloramphenicol.

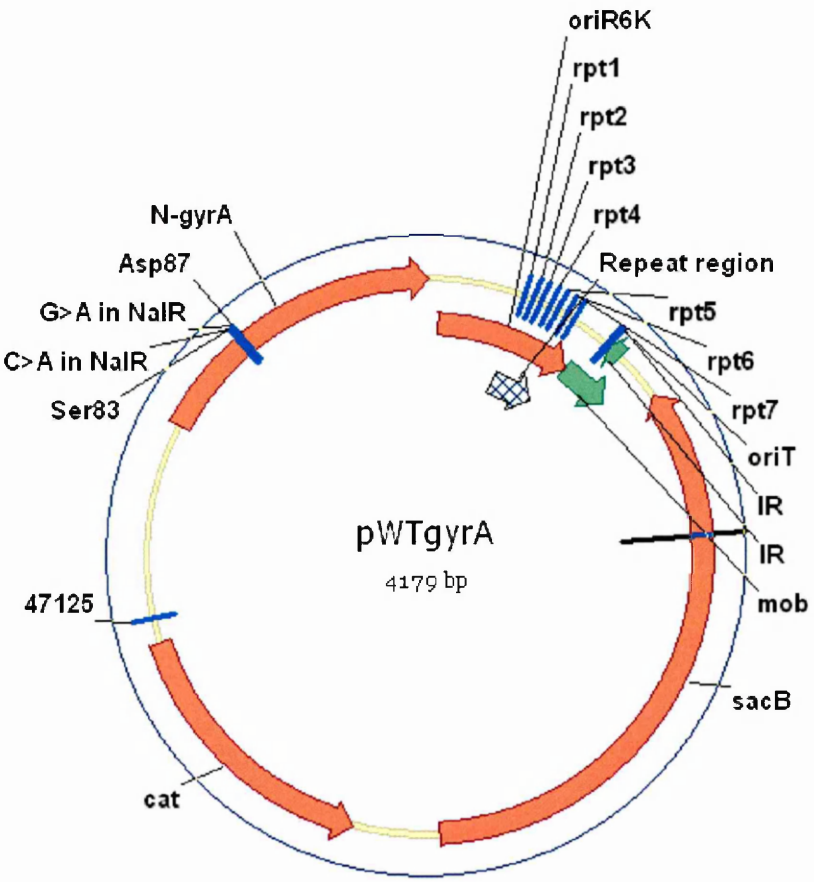


Figure 2.1 A schematic representation of the plasmid vector pJCB12 containing a mutant *gyrA* loci containing an amino acid substitution

pJCB12 and derivative plasmids contain the *cat* gene for chloramphenicol resistance and the *sacB* gene which is responsible for the counter-selection of recombinants. oriR6K (R6K origin) is a replication origin for recipients. Rpt (Repeats) 1 to 7 are DNA polymerase binding sites for replication. The mob (mobilization) region is necessary for plasmid mobilization into recipient strains. IR (Inverted Repeat) is the inverse sequence of the polymerase binding site. *oriT* is the origin of transfer which is for functional transfer of the plasmid from a host to a recipient during bacterial conjugation.

2.2 Identification

All *S.* Typhi isolates were isolated from blood, bone marrow or stool cultures from patients who were admitted to the hospitals in the locations outlined previously. The process was firstly to identify the bacteria at the time of sample collection using routine laboratory methods including screening bacteria on MacConkey agar (MC) (Oxoid, UK) and Xylose Lysine Deoxycholate agar (XLD) (Sigma-Aldrich, Switzerland). *S.* Typhi isolates were recognized as a colourless and transparent colony on MacConkey agar (Figure 2. 2a) as *S.* Typhi cannot utilize lactose as a carbohydrate source. On XLD agar, *S.* Typhi is seen as red colonies with a black centre (Figure 2.2b). This is the result of pH change because *S.* Typhi strains exhaust the xylose and decarboxylate the Lysine. Therefore, *S.* Typhi strains produce sulphide on XLD agar which is recognized by a blackening in the colony centre. All isolated colonies were then subjected for the short biochemical and serological testing.

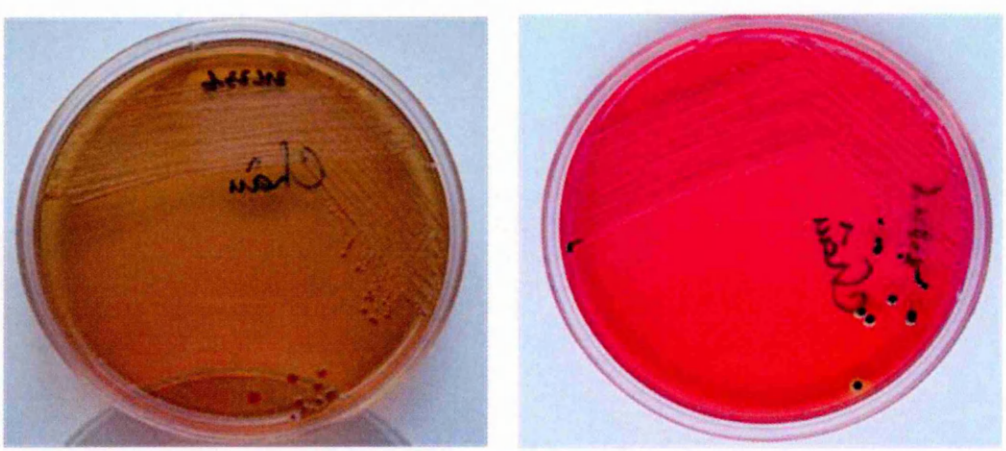


Figure 2.2 *S.* Typhi morphology on selective media

(a) *S.* Typhi morphology on MacConkey agar, seen as a colourless and transparent colony.
(b) *S.* Typhi morphology on XLD agar, *S.* Typhi can be recognized as red colonies with black centre.

2.2.1 Serological agglutination method

Serological agglutination was carried out using specific agglutination antisera. *S.* Typhi strains were defined when positive agglutinations occurred between cell suspension and poly O, O_9 and Vi (Murex, Dartford, UK) antisera. If O_9 was positive but Vi was negative, the colony was then inoculated and re - tested after grow in 1 mL of Luria Bertani media overnight.

One drop of agglutinating sera of *S.* Typhi was placed on a clean glass slide. One colony of test strain which was picked with a loop from nutrient agar plate was mixed slowly with the agglutinating serum. When fully mixed, the slide was rotated for 5-10 seconds. Agglutination was observed by the naked eye. Positive and negative controls were also performed concurrently.

2.2.2 Short biochemical test

Tests were performed in parallel using specific media; *S.* T yphi was identified as organisms with weak sulphite production, glucose but not lactose fermentation, no gas production in Kliglar iron agar, citrate and urea and indole negative and motility positive (Figure 2. 3). Isolates with unclear biochemical characteristic were confirmed by the API 20E (Biomerieux, Paris, France).

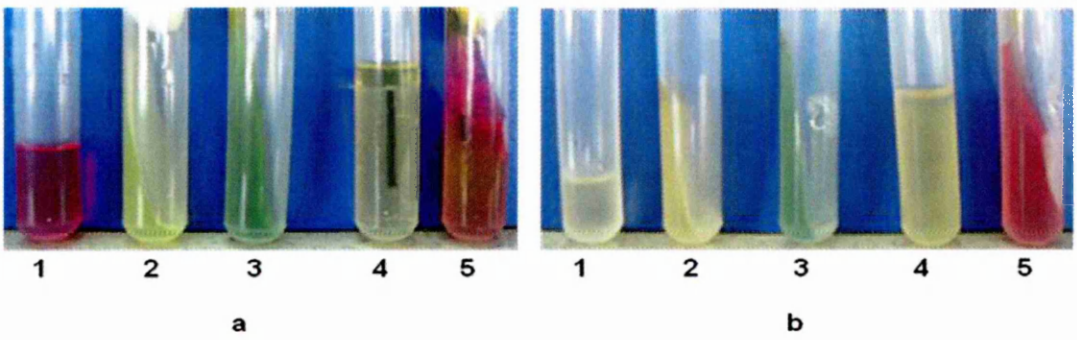


Figure 2.3 A short set of the biochemical testing for *S.* Typhi identification

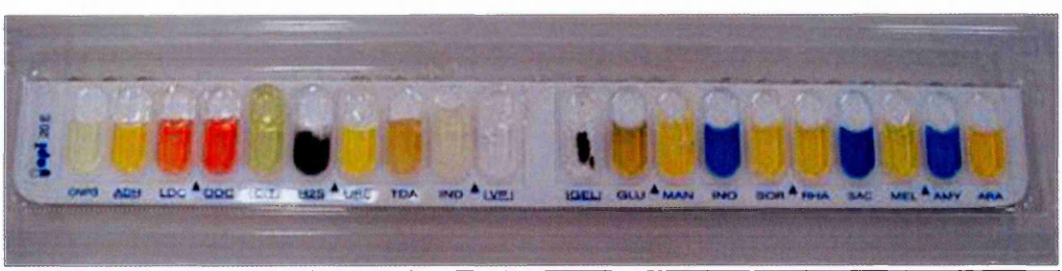
(a) Biochemical testing consistent with the identification of *S.* Typhi. (b) The same biochemical testing as in (a) but without bacterial inoculation. Tests are as follows, (1) Methyl red test; methyl red is added after an overnight bacterial culture, if the organism can ferment glucose, acid is produced and the media turns red, *S.* Typhi ferments glucose and therefore, generates a positive result. (2) Urea utilization; the yellow colour will turn to pink after a positive reaction. *S.* Typhi cannot not use urea as a substrate for metabolism and is therefore, negative. (3) Citrate utilization; *S.* Typhi can be distinguished from other *Salmonella* by not being able to utilize citrate. The green will turn to blue after a positive reaction. (4) Motility test, *S.* Typhi is motile in liquid media. The motility test detects hydrogen sulphide production from a sulphur source medium. Motile organisms produce hydrogen sulphide which reacts with ferrous sulfate and is precipitated as a black line in the media. (5) The KIA, Alkaline / acid formation test; *S.* Typhi can utilize glucose but not lactose, in KIA media, *S.* Typhi changes the butt colour to yellow (acidity) due to the acid production, the same is not observed in media due to ammonia maintaining the overall alkalinity. Additionally a small amount of H_2S can be observed in the butt.

2.2.3 The API 20E

The API 20E is a biochemical identification system for the *Enterobacteriaceae.* The system consists of a plastic strip with 20 microtubes containing dehydrated substrate of ornithine, citrate, hydrogen sulphide, urea, tryptophane, indole, voges-proskauer, gelatin glucose, manitol, inositol, sorbitol, rhamnose, saccharose, melibiose, amygdaline and arabinose (Figure 2. 4). The procedure was according to the manufacturer's recommendations (Biomerieux). Briefly, test substrates were reconstituted when inoculated with a suspension of the test organism in sterile saline. After inoculation the cupula sections of arginine, lysine, ornithine, H_2S, and urea microtubes were overlaid with mineral oil. The incubation box was prepared by putting 5 mL of sterile water into the honey combed wells of the tray. The strip was placed in an incubation tray

containing water and the colony emulsified in sterile saline was transferred to the cupules After 24 hour incubation at 37 °C, the strip was read by referring to the interpretation table. Reagents were added into the VP, TDA, indole, nitrite cuptules and the results were recorded. The 21 test results, which include oxidase detection which detected cytochrome c oxidase, is converted to a seven digit profile using the octal coding system following the manufacturer's instruction. Identification was made through the analytical profile index by the use of a differential chart. Oxidase test was performed separately.

By API 20E, *S.* Typhi was detected while giving positive reaction to LDC and H_2S. It utilized glucose (GLU), manitol (MAN), sorbitol (SOR), melibiose (MEL) and arabinose (ARA) as metabolite resources, and *S.* Typhi was negative to oxidase test. *S.* Typhi was coded as 4404540 by the definition manual (Bio Merieux) (Figure 2. 4).



ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	Oxid -ase
-	-	+	-	-	+	-	-	-	-	-	+	+	-	+	-	-	+	-	-	-

Figure 2.4 Representative biochemical characteristic of *S.* Typhi using API 20E test

The API20E test strip includes reactions for, ONPG (b-galactosidase), ADH (decarboxylation of the amino acid arginine by arginine dihydrolase), LDC (decarboxylation of the amino acid lysine by lysine decarboxylase), ODC (decarboxylation of the amino acid ornithine by ornithine decarboxylase), CIT (utilization of citrate as sole carbon source), H_2S (hydrogen sulfide), URE (urease), TDA (tryptophan deaminase), IND: (tryptophanase, Indole is detected by addition of Kovac's reagent), VP (Voges-Proskauer test for the detection of acetoin (acetyl methylcarbinol) produced by fermentation of glucose), GEL (gelatinase which liquefies

gelatin) , GLU (fermentation of glucose), MAN (mannose), INO (inositol), SOR (fermentation of sorbitol) RHA (fermentation of rhamnose), SAC (fermentation of sucrose), MEL (fermentation of melibiose), AMY (fermentation of amygdalin), ARA (fermentation of arabinose) and OX (cytochrome oxidase).

2.3 Growth supplements and storage

Isolates were cultured on nutrient agar (Oxoid, UK) and stored in Brain Heart Infusion (BHI) (Fisher Scientific, UK) with 15% Glycerol at -30 °C for further studies. Luria-Bertani (LB) broth (Sigma-Aldrich, Germany) was used in bacterial growth curves and molecular experiments. LB was made by suspending 20g LB powder in 1L distilled water and autoclaving for 15 minutes at 121 °C.

When culturing strains containing plasmids and attenuated *S.* Typhi strains, media were supplemented with chloramphenicol (Sigma) (15 μg / mL) and "aromix" (40 μg / mL L-phenylalanine, 40 μg / mL L-tryptophan, 10 μg / mL ρ - aminobenzoic acid, 10 μg / mL 2, 3-dihydroxybenzoic acid and 40 μg / mL L-tyrosine). When selecting attenuated *S.* Typhi strains which did not harbor the suicide vector, medium was supplemented with 5 % sucrose and $NaCl_2$ was excluded from the recipe [118].

Oxgall, dehydrated fresh bile salts, is a known culture medium for isolation of *Salmonella* from blood [135]. Bile salts have a detergent - like activity, which can permealize bacterial membranes and can eventually lead to membrane collapse and cell damage [136-138]. Eight percent concentration of oxgall is the natural concentration of bile salts in the human gall bladder [139]. Although the tolerance of *S.* Typhi to oxgall was demonstrated to influence the persistence of *S.* Typhi in the chronic carrier state [140], in this study, oxgall (Difco) supplemented in LB aro was used as a stress factor in order to evaluate the growth ability of the reconstructed *S.* Typhi mutant under a growth pressure. The following concentrations of oxgall were applied for screening the most

appropriate concentration of oxgall for differentiating growth abilities of the reconstructed mutants. The various concentrations of oxgall supplemented in LB aro were 0.0193 %, 0.186 %, 0.375 %, 0.625 %, 1.25 %, 2.5 %, 5 %, 6 %, 7 %, 8 %, 9 %, 10 %, 11 % and 12 %.

2.4 Antimicrobial susceptibility

Antimicrobial containing disks and strips were supplied by Difco (UK) and AB Biodisk (Solna, Sweden), respectively. Nalidixic acid, ofloxacin, ciprofloxacin, gatifloxacin, chloramphenicol and ampicillin powders were supplied by Sigma-Aldrich (Germany).

2.4.1 Disk diffusion method

Antimicrobial susceptibility testing was performed at the corresponding concentrations to five non - fluoroquinolones; ampicillin (10 μg), chloramphenicol (30 μg), trimethoprim-sulfamethoxazole (1.25 / 23.75 μg), ceftriaxone (30 μg) and azithromycin (15 μg); one quinolone (nalidixic acid (30 μg) and three fluoroquinolones; ofloxacin (5 μg), ciprofloxacin (5 μg) and gatifloxacin (5 μg) (Oxoid, Basingstoke, UK) by disc diffusion according to Clinical Laboratory Standards Institute (CLSI) methods [141], and interpreted following CLSI guidelines [141]. No CLSI MIC breakpoints of azithromycin for enterobacteriacea are currently recommended according to these guideline, therefore, to analyse AZM susceptibility for *S.* Typhi we used the current MIC breakpoints described for *Staphylococcus* spp. [141]. An equivalent bacterial suspension to a 0.5 McFarland standard was used for disc diffusion method. The control strains used for all susceptibility tests were *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213. The discs are impregnated with a specified amount of the antimicrobial agent and are placed on the Mueller-Hinton (MH) agar (diameter 90 mm, thickness 4 mm) by the modified Kirby-

Bauer disc diffusion method [142]. The plate was seeded uniformly with bacteria. Four to seven antimicrobial disks were applied onto the plate after allowing the surface moisture to be absorbed. After incubation for 24 hours, the size of the zones of inhibition of growth produced by the antimicrobial agents diffusing from the discs into the surrounding agar was measured [141].

2.4.2 E - test

The MIC is the most commonly used parameter to describe the efficacy of an antimicrobial agent against a bacterial strain. This is an *in vitro* measure reflecting the efficacy of a constant antimicrobial exposure to a specified bacterial inoculum after an incubation period of 16 to 20 hours. The MIC is an estimate of the susceptibility of a bacterial strain to an antimicrobial that can guide the choice of an appropriate antimicrobial therapy [141].

The MIC was determined by E - test (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. Similar to the disk diffusion method an MH plate was swabbed with the organism and allowed to dry before E - test strips were placed on the surface. Plates with strips containing the tested antimicrobials (two strips per plate) were incubated for 24 hours and the results were interpreted following CLSI guidelines [141].

Multidrug resistance (MDR) of isolates was defined as resistance to chloramphenicol (MIC $\geq$ 32 μg / mL), ampicillin (MIC $\geq$ 32 μg / mL) and trimethoprim-sulfamethoxazole (MIC $\geq$ 8 / 152 μg / mL). Nalidixic acid resistance was defined as MIC $\geq$ 32μg / mL. The breakpoints for ofloxacin and gatifloxacin were $\leq$ 2 μg / mL susceptible and $\geq$ 8 μg / mL resistant, for ciprofloxacin $\leq$ 1μg / mL susceptible and $\geq$ 4

μg / mL resistant. The breakpoints for ceftriaxone were ≤ 8 μg / mL susceptible and ≥ 64 μg / mL resistant [141]. All tests were performed at the Hospital for Tropical Diseases (HTD), Ho Chi Minh City, Viet Nam, except for the isolates from Nepal, which were tested at Patan Hospital, Kathmandu, Nepal, using identical methods.

2.5 Biological cost evaluation

2.5.1 Growth curves assay

The fluorostar system is a spectrophotometer that measures UV based absorbance (Halmington, Switzerland). The Omega microplate reader is capable of capturing an absorbance spectrum (220 - 850 nm) at a resolution of 1 nm. This system was used for monitoring bacterial growth dynamics.

Three individual colonies were grown in 5mL LB (supplemented with 1 % aromix when working with BRD 948 derivatives). One microliter of overnight culture was inoculated in 200 μl of LB (LB aro) or LB supplemented with oxgall in Nunc96 plate (ThermoFisher Scientific, US). The growth rate was performed in BMG Fluorostar Omega system (Halmington, Switzerland) using below absorbance protocol. Each culture was incubated in 5 random positions in the plate at 37 ^{o}C with 300 rpm shaking speed and 60 seconds additional shaking before each cycle. Optical density readings at 600 nm were performed for each well every 10 minutes over 24 hours and plotted against time. The resulting data was analyzed using Omega software supplied.

2.5.2 Competitive assay

The relative competitive fitness of bacteria was quantified experimentally with the use of competition assays [143].

The nalidixic acid susceptible ancestor (*S.* Typhi BRD 948) and nalidixic acid resistant *S.* Typhi strains (engineered mutants) were mixed and competed directly in a non - selective medium (LB aro). The *S.* Typhi BRD 948 strain and the engineered mutant were initially grown separately in 10 mL of LB aro at 37°C with shaking to stationary phase (10^8 CFU / mL). One microliter of each was co-inoculated into a new 10 ml LB aro, and 10 µl of the mixed overnight culture was continuously transferred to a fresh 10ml LB aro every day for 15 days. Two methods were used to determine the relative fitness of nalidixic acid resistant *S.* Typhi, namely colony counting and quantitative pyro-sequencing.

Colony counting of the mixture was performed by serial ten-fold dilution in MRD and then plating. Fifty microlitres of each solution was diluted serially in 450 µl of MRD in 96-well microtitre plate. After mixing by pipetting thoroughly, 20 µl of 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} dilutions were dropped onto both LB aro plates and LB aro plates supplemented with 20 µg / mL nalidixic acid. All plates were incubated at 37°C overnight. Plates with between 10 and 100 colonies were counted. The counting was carried out at the beginning of the experiment (Time 0) and every 24 hours for 15 days.

Additionally, 1mL of overnight culture from the competition assay was subjected to DNA extraction (Section 2.7.1.3). DNA was directly applied for quantification of the growth rate of each strain in the mixture using pyrosequencing technique, by monitoring the proportion of individual SNPs. (Section 2.7.5.2). Competition experiments were performed in triplicate and independently.

2.6 In vitro time - kill analysis

Time - kill experimentation is usually applied to evaluate the pharmacodynamic of fluoroquinolones on bacterial strains [116]. This method allows measuring the effects of different concentrations of an antimicrobial at definite time points. In this study, time - kill experiments were applied to measure the efficacy of fluoroquinolones on different *S.* Typhi mutants which conferred nalidixic acid resistance and reduced susceptibility to fluoroquinolone in *S.* Typhi strains.

All time - kill experiments were performed in duplicate. Ofloxacin powder was purchased from Sigma, Steinheim, Germany and gatifloxacin powder was purchased from Bristol-Myers Squibb, New Brunswick, USA. Three *S.* Typhi colonies were selected and inoculated in 10 mL Mueller-Hinton broth (Oxoid, UK) and were incubated at 37 °C for 15 to 18 hours. 100 µl of the overnight culture was inoculated into 10 mL of Mueller-Hinton broth and incubated at 37 °C for one hour to give 2 x 10^6 CFU / mL. 10 mL of Mueller-Hinton broth containing ofloxacin or gatifloxacin at 32x MIC were added at time 0 to give a final concentration of 16 x MIC; serial two fold dilutions were used to obtain 8x, 4x, 2x and 1x MIC. The growth control contained no antimicrobial. The cultures were incubated at 35 - 37 °C for 24 hours. Viable counts were measured immediately prior to the addition of the antimicrobial and at 30 minutes and 1, 2, 4, 6, 8 and 24 hours after the addition of the antimicrobial. Viable counts were performed on nutrient agar plates following serial ten-fold dilution in MRD broths [144]. The lower limit of detection was 10 CFU / mL.

2.7 Molecular methods

2.7.1 Genomic DNA isolation

DNA was extracted either by phenol-chloroform (or CTAB) method [145], Promega kit (Promega, Madison, WI), or rapid boiling, which depended on each experimental requirement or the availability of DNA extraction kits.

2.7.1.1 Genomic DNA extraction using Phenol-Chloroform or CTAB (Cetyl Trimethyl Ammonium Bromide) method

All *S.* Typhi strains and other bacterial strains from which DNA was extracted were grown overnight in 5 ml of LB broth at 37°C with agitation. 1.5 mL of the overnight bacterial culture was centrifuged, the supernatant was removed and the resulting pellet was re-suspended in 567 µL of TE buffer (pH 8); 30 µL of 10 % SDS and 3 µl of 20 mg / mL proteinase K. The solution was mixed and incubated for 1 hour at 37°C. 100 µl of 5M NaCl was added and the solution was mixed thoroughly. To this solution, 800 µl of CTAB / NaCl solution was added and incubated for 10 minutes at 65 °C. An equal volume of chloroform / isoamyl alcohol was added and mixed thoroughly. The solution was centrifuged in a microfuge at 13,000 rpm for 5 minutes. In samples where no interface was formed after centrifugation, the samples were mixed again and subjected to further centrifugation. The aqueous layer was transferred into a clean microfuge tube and an equal volume of phenol : chloroform : isoamyl alcohol was added, gently mixed and centrifuged for 5 minutes at 13,000 rpm. The upper layer was collected and the nucleic acid was precipitated with 0.6 volumes of isopropanol after centrifugation at 14,000 rpm for 15 minutes. The nucleic acid was stored at -70 °C for an hour or at -20 °C over night to yield more DNA. The DNA was washed in 70 % ethanol twice and dried for 10 minutes at 50 °C. The DNA pellet was re-suspended in 50 µl of TE buffer

and 1µl of DNA solution was analyzed by electrophoresis on a 0.8 % agarose for purity and concentration.

2.7.1.2 Genomic DNA extraction using Promega kits

A single colony was inoculated in 1.5 mL of LB broth and incubated overnight at 37 °C with shaking at 300 rpm to reach 10^8 CFU / mL. One milliliter of the bacterial culture was transferred to a clean eppendorf and centrifuged the tube at 13,000 rpm for 2 minutes. The supernatant was removed and bacterial pellet was used for DNA extraction using Wizard genomic DNA purification kit (Promega, USA) according to the manufacturer's recommendations. The extracted DNA was stored at –20 °C until required.

2.7.1.3 DNA extraction using the boiling technique

For screening for the presence of plasmid-mediated quinolone resistance gene including *qnrA* and *qnrS* genes, a quick DNA extraction method was applied. This method allows harvesting a small amount of DNA that could be subjected to PCR. However, the DNA cannot be stored for a long time use or for further molecular investigations because of the lack of purity and degradation. A single colony was inoculated in 1 mL of LB broth and incubated overnight at 37 °C with shaking at 3,000 rpm. Five hundred microliters of the culture was denatured in a 1.5 mL eppendorf at 100 °C for 10 minutes, five hundred microliters of chloroform was added and mixed thoroughly. After centrifugation at 13, 200 rpm for 10 minutes, DNA in the upper layer was used directly applied for PCR.

2.7.2 Polymerase chain reaction (PCR)

The PCR methods were applied to amplify DNA targets. In general, all PCR were performed using standard PCR programme with 3 steps, denaturation, annealing and elongation. Bioline *Taq* pol ymerase (Bioline) was employed for conventional PCR reactions for all templates except large target fragments which were predicted to be more than 20 kb in length. For long templates, the Expand Long Template PCR system was used (Roche Lifesciences).

Four PCR methods were applied in this study depended on the purpose of each experiment. There are subtle differences in the principle used between conventional PCR, CEQ sequencing system (Beckman Coulter, USA) and PCR for pyrosequencing (Biotage, Sweden). Whilst dideoxynucleotide (ddNTP) is used in PCR for sequencing, a primer labeled with biotin at 5′ end is applied in PCR for pysosequencing. Multiplex PCR and colony PCR were conducted to simultaneously amplify more than one target and for rapid screening of PCR products, respectively. All PCR amplifications were carried out using the DNA Engine2Tetrad thermal cycler (Biorad, USA).

2.7.2.1 Conventional polymerase chain reaction (PCR)

Oligonucleotide primer pairs for detection mutations and deletion fragments are presented in Table 2.2 and Table 2.3. PCR amplifications of *gyrA* (347 bp), *gyrB* (345 bp), *parC* (270 bp), *parE* (240 bp) and PCR for investigating the deletion haplotype representatives were performed with 30 cycles of denaturation at 92 °C for 45 seconds to one minute, annealing at 45 - 62 °C for 45 seconds to one minute and extension at 74 °C for 45 seconds to 2 minutes, followed by a final extension step at 74°C for 2 to 5 minutes.

Table 2.2 List of primers for *gyrA, gyrB, parC* and *parE* genes amplification

Gene	Primer	Primer sequence (5'→3')	References
GyrA	GYRA/P1	TGTCCGAGATGGCCTGAAGC	[146]
	GYRA/P2	TACCGTCATASGTTATCCACG	
GyrB	StygyrB1	CAAACTGGCGGACTGTCAGG	[147]
	StygyrB2	TTCCGGCATCTGACGATAGA	
ParC	StmparC1	CTATGCGATGTCAGAGCTGG	[148]
	StmparC2	TAACAGCAGCTCGGCGTATT	
ParE	StmparE1	TCTCTTCCGATGAAGTGCTG	[148]
	StmparE2	ATACGGTATAGCGGCGGTAG	

Table 2.3 List of primers used in PCR - based typing for Insertion - deletion investigation

Name of deletion	Primer sequence (5'→3')	
	Forward	Reverse
A	AGCGATGTGATGATCAGGATT	AATGGCGTGTTCAGTGGATT
B	TCCGTCTCTTTCTCCAGC	AATTGATGCTGCTGCTGGACG
C	ACGGGTGAAATACTCGAACG	CACCAAGCAGATTGTTCAGG
D	CGCTATTTTTTCCGCCCATGC	TAATAACATCGGCGTGCCG
E	CCGTCGCCAAAGTGACGC	CCGTTGAATCGGAAGTAATAATCG
F	AAGCAAATGCTTAGCACCAC	CAATGCATAAAGTTAATTTAATCAGGA
K	ATGGGTGAGCGCCTCTTTGG	GACTGGCTGGACATTTTGTG
N	ATGTTTCATGTGTGGGTAGGGTTGCC	AAGAATGCCCATTGAGCGG
Q	CAACACCCGTGCGGACGAT	AGCTTACTTCCGGCTCCGAC
S	TTGGTGATAAAATTGGCTCGGG	AAAGAATGGAAACCAGAGTTTCC
H	GTGGCAAAACAACGCATCG	CGGTGGAGTTAGTGATGCTGA

2.7.2.2 PCR for screening *qnr* genes

The positive control used in for *qnr* genes detection was a *Citrobacter spp.* isolate (identified by API20E) harbouring both *qnrA* and *qnrS* genes which was confirmed by sequencing PCR products [149]. The PCR primer pairs for *qnrA* and *qnrS* identification were qnrAF (5′- TCAGCAAGAGGATTTCTCA - 3′) and qnrAR (5′- GGCAGCACTATTACTCCCA- 3′); and qnrS1 (5′ - ATGGAAACCTACAATCATAC - 3′) and qnrS2 (5′- AAAAACACCTCGACTTAAGT - 3′). Twenty five microliters PCR reactions for *qnrS* gene detection was used containing, 2.5 µl buffer, 1 µl of 50 mM $MgCl_2$ 1 µl of 5 µM each primer, 2.5 µl of 2.5 mM dNTPs, 0.8 µl Bioline *Taq*

polymerase, 2 µl DNA which was extracted by boiling technique (Section 2.7.1.3) and 14.2 µl distilled water. The 25 µl PCR mixture for *qnrA* gene detection included 2.5 µl 10X buffer, 1 µl of 50mM $MgCl_2$, 0.05 µl of 100µM each primer, 2.5 µl of 2.5mM dNTPs, 0.8 µl Bioline Taq polymerase, 2 µl DNA which was extracted by boiling technique and 16.1µl distilled water. PCR amplification was performed using 34 cylces of denaturation at 94°C for 45 seconds, annealing at 45 °C (for *qnrS* gene) and 53°C (for *qnrA* gene) for 45 seconds and extension at 72°C for 45 seconds, followed by a final extension step at 74°C for 5 minutes.

2.7.2.3 PCR for sequencing

The principle of PCR for sequencing is based on the fact that the deoxynuxleotides (dNTP) and dideoxynucleotides (ddNTP) are co-present in the PCR reaction and each ddNTP is labeled in four different fluorescence colours. In addition, the -OH group at C3 of ddNTP is replaced by -H, therefore the elongation step of the PCR reaction is stopped when a ddNTP is incorporated on the adjacent on the template.

Purified PCR products were subjected for the sequencing reactions. Both strands of each DNA sample were independently sequenced; therefore, two PCR reactions were carried out using either forward or reverse primers (Table 2.2). Twenty microliters PCR reaction included; 4 µl DTCS master mix, 2.5 µl 1 µM primer, 2 µl of 10 ng / µl DNA and 11.5 µl distilled water. PCR was performed with 30 cycles of denaturation at 96 °C for 20 seconds, annealing at 55 °C (for *gyrA* gene) and 62 °C (for *gyrB, parC* and *parE* genes) for 20 seconds and extension at 60 °C for 2 minutes.

2.7.2.4 PCR for pyrosequencing

The PCR reaction for pyrosequencing was carried out with a primer pair in which a forward primer was labeled with biotin at the 5′ end. A hundred microliter PCR reaction was performed including 1.25U Hotstart *Taq* polymerase, 1X NH_4 buffer (10X), 1.5mM $MgCl_2$ (25 mM), 200 µM dNTPs (2.5 mM), 10 pM gyrApyro-F (1 µM) (5′-TGGGCAATGACTGGAACAAAG - 3′), 10 pM GYRA / P2 Biotin – TACCGTCATAGTTATCCACG - 3′ (1 µM) and 400 ng of DNA which was adjusted to 100 µl with distilled water. PCR programme for *gyrA* (291 bp) was performed with 25 cycles of denaturation at 94 °C for 5 minutes, annealing at 55 °C for 1 minute and extension at 72 °C for 1 minute, followed by a final extension step at 72 °C for 10 minutes. The PCR programme for *parC* was 94 °C for 5 minutes, 94 °C for 30 seconds, 57 °C for 30 seconds, 72 °C for 1 minute and 72 °C for 5 minutes. PCR products were visualized on 1 % agarose gel before being subjected to the pyrosequencing procedure.

2.7.2.5 Expand long template PCR

Expand Long Template PCR system (Roche, Germany) was used to amplify large target sequences, as per the manufactures recommendations.

2.7.2.6 Colony PCR

The multiplex PCR and colony PCR amplifications were applied to screen the presence of nucleotide sequences in a PCR based typing method or to examine genetic constructs. PCR reactions were prepared including 20 µl Platinum ® PCR Supermix (Invitrogen, USA) and 1µl of a solution which was a colony suspended in 100 µl distilled water (or 200 ng of stock DNA). Standard PCR programme was performed with 29 cycles of denaturation at 95 °C for 10 seconds, annealing at 55 °C for 1 minutes and extension at 72 °C for 1 minute 20 seconds, followed by a final extension step at 72

°C for 3 minutes. The primer pairs using in the reconstruction of *gyrA* and *parC* genes are listed in Table 2. 4.

Table 2.4 List of primers for reconstructed mutations on *gyrA* and *parC* genes

Name	Sequence (5′→3′)	Target
47125	TTCATTATGGTGAAAGTTGGAACC	screening for CVD908-htrA parC::pWT-parC15 derivatives
Cat-12	CCATAATCAGTCGACGGTATCGATAAGCTT	Amplification pJCB12 fragment
GyrA-07	GGGTCGACTGATTATGGTTTATGCCTCC	construction of all *gyrA* mutations
GyrA-10	GCGCATGCCACGACCGGTACGGTAG	construction of all *gyrA* mutations
GyrA-11	TCGCGTCAGCTTCAACTTCC	screening for BRD 948 *gyrA*::pWT-*gyrA* derivatives
gyrA-23	CTCTTCAGCTCTTCCTCAAT	construction of all *gyrA* mutations
GyrA-31	CTATTCGGGAACTTTGTACCC	construction of all *gyrA* mutations
GyrA-45	AACGATGGTGCCATACACTGCGGAATC	construction of *gyrA* S83F and D87G
GyrA-46	TTCCGCAGTGTATGGCACCATCGTTCG	construction of *gyrA* S83F and D87G
GyrA-47	AACGATGGTGCCATACACTGCGAAATC	construction of *gyrA* S83F and D87G
GyrA-48	TTTCGCAGTGTATGGCACCATCGTTCG	construction of *gyrA* S83F and D87G
GyrA-49	ATACCGTCGACTGATTATGGTTTATGCCTCC	construction of *gyrA* D87A mutation
GyrA-50	CGATGGTGGCATACACTGCGGAAC	construction of *gyrA* D87A mutation
GyrA-51	CGCAGTGTATGCCACCATCGTTCG	construction of *gyrA* D87A mutation
GyrA-52	CGATGGTGGCATACACTGCGAAATC	construction of *gyrA* D87A mutation
GyrA-53	CGCAGTGTATGCCACCATCGTTCG	construction of *gyrA* S83F and D87A
GyrA-54	GGCGATTCCGCAGTGTATGC	construction of *gyrA* S83F and D87A
ParC-18	GCGGTCGACGTTGGCGTGGTGAATAG	construction of all *parC* mutations
ParC-21	CGCGCATGCGGCAACGCGGTGATCA	construction of all *parC* mutations
ParC-23	GCCAGCAGTCAAACTTTTGG	construction of all *parC* mutations
ParC-25	CCAGACCGATCATGTTCAGG	construction of *parC* S80I mutation
ParC-27	ATAGCAGGCGATGTCGCCATG	construction of *parC* S80I mutation
ParC-28	CATGGCGACATCGCCTGCTAT	construction of *parC* S80I mutation
ParC-29	TATCACCCGCATGGCGACAT	construction of *parC* S80I mutation
R6K-01	GTGACACAGGAACACTTAACGGC	screening for BRD 948parC::pWT-*parC* derivatives
R6K-03P	P-CCATGTCAGCCGTTAAGTGTTC	amplification pJCB12 fragment

2.7.3 PCR product purification

Depending on the requirement of further investigations, PCR products were purified using QIAquick PCR purification kit following the manufacturer's instructions (QIAGEN GmbH, Germany).

2.7.4 DNA quantification

2.7.4.1 Quantification of DNA by spectrophotometers

The absorbance at 260 nm was determined using a Nanodrop ND - 1000 spectrophotometer (NanoDrop technologies Inc., USA) which is a cuvette free spectrophotometer. A drop of distilled water was used as a blank. The concentration of DNA was calculation using the following formula:

$$c = (A_{260} \times 50)/0.1$$

Where **c** is the nucleic acid concentration in ng / microliter, $\mathbf{A_{260}}$ is the absorbance in AU, and the path length is 0.1 cm. The wavelength-dependent extinction coefficient of double stranded DNA is 50 ng - cm / µl.

2.7.4.2 Quantification of DNA using agarose gel electrophoresis

One microliter of DNA sample was mixed with 5 µl of 6x loading buffer and samples were loaded into the wells. Two and half microliters of controls of the Precision Molecular Mass standard (BioRad, USA) were loaded simultaneously. The concentrations of the standard are 10 ng for 100 bp band and 20 ng, 50 ng, 70 ng and 100 ng for 200 bp, 500 bp, 700 bp and 1000 bp, respectively.

2.7.5 Sequencing

2.7.5.1 Conventional (Sanger) sequencing

Sequencing reaction was performed by the CEQ DTCS - Quick Start Kit and analyzed using CEQ2000XL software (Beckman Coulter, USA). A sequencing reaction involves 3 steps including preparation of the DNA sequencing reaction (section 2.7.2.3), ethanol precipitation of sequencing products and sequence data analysis.

Excess PCR reagents were removed from the PCR products by ethanol precipitation in the presence of sodium acetate. The precipitation reaction included 4 µl of stop solution (3M NaOAc and 100 mM EDTA) and 1µl of 20 mg / mL glycogen (supplied in DTCS Quick Start kit) and 20 µl of a sequencing reaction. The solution was mixed thoroughly before and after the addition of 60 µl of cold 95 % ethanol. The DNA pellet was harvested after centrifuging at 14,000 rpm for 15 minutes and washed twice with 200 µl of cold 70 % ethanol. For each wash, the eppendorf was centrifuged immediately at 14,000 rpm for 2 minutes. The DNA pellet was allowed to dry for 15 minutes before rehydrated in 40 µl of sample loading solution for at least 15 minutes (supplied in DTCS Quick Start kit, USA). Finally, the DNA product was subjected for an electrophoresis in the CEQ 8000 capillary sequencer, and the result was analyzed using CEQuence Investigator_CEQ2000XL.

2.7.5.2 Pyrosequencing

Pyrosequencing is a DNA sequencing technique that is based on the transformation of released pyrophosphate (PPi) during DNA synthesis into measurable light. Visible light generated is proportional to the number of incorporated nucleotides. During the nucleic acid polymerization, a PPi is released as a result of nucleotide incorporation by polymerase. The PPi released is subsequently converted to ATP by ATP sulfurylase,

which provides the energy to luciferase to oxidize luciferin and generates light. Because the added nucleotide is known, the sequence of the template can be determined [150]. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as a peak in a pyrogram.

In this study, pyrosequencing was applied for detection and quantification of SNPs in the *gyrA* gene (Figure 2.5). The 3′- end of a primer was designed to hybridize to one or several bases before the polymorphic position. PCR products were sequenced using Pyro Gold SQA sample preparation kit and a PSQ 96MA analyzer (Biotage, Sweden). Generally, biotinylated PCR products were immobilized onto streptavidin-coated Sepharose beads (Streptavidin Sepharose HP, Amersham Biosciences, Sweden) in a sealable 96 - well PCR plate by adding 40µl of the binding buffer - Streptavidin Sepharose bead mix (3µl per sample PSQ 96, PSQ 96MA and 37 µl Binding buffer) into 80µl of a well-optimized PCR reaction. The plate was sealed and incubated with shaking at room temperature for 5 minutes with agitating constantly to keep the beads dispersed. Beads containing immobilized DNA templates were captured on the filter probes, washed in 70 % ethanol, denatured in denaturation solution, washed again in washing buffer then released the beads in a PSQ HS 96 plate low, prefilled with Annealing buffer containing 40 µl of 3.5 pM pyroGDSD-3 (5′ - CGGTAAATACCATCCCCA - 3′) per well. Labeled nucleotides were added by cyclic dispensation. Sequencing results were analyzed using the IdentiFire software (Biotage, Sweden).

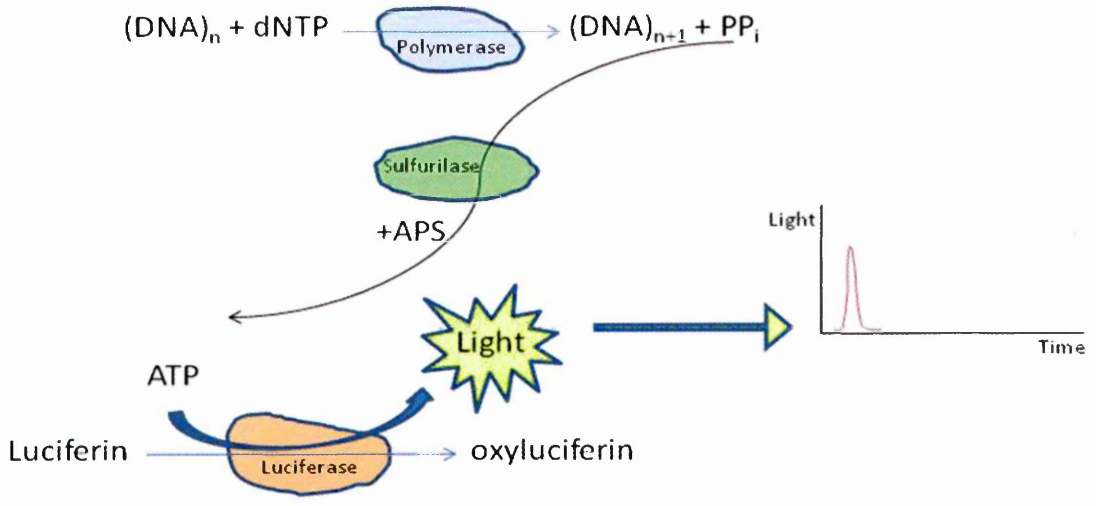


Figure 2.5 The principle of pyrosequencing methodology

The pyrosequencing procedure utilizes 3 reactions. (1) The DNA synthesis releases pyrophosphate (PPi), (2) The conversion of PPi to ATP by ATP sulfurylase, (3) The oxidization of luciferin and generation of light using the energy from reaction (2). Then, the light is detected by a charge coupled device (CCD) camera and seen as a detectable peak.

2.7.6 Reconstruction of mutation on *gyrA* and *parC* genes

2.7.6.1 The *gyrA* and *parC* mutations were reconstructed in *S.* Typhi BRD 948 by the allelic exchange method.

The allelic exchange for integrating gene into the chromosome occurs inside a cell following a two-step procedure. Firstly, the gene is cloned into a plasmid and the entire plasmid is integrated into the chromosome by a single-crossover between the homologous genes thereby producing a chromosomal duplication. Second, the chromosomal duplication is segregated by homologous recombination between the flanking direct repeats, ultimately leaving one of the copy of the genes on the chromosome (either the wild type or the mutant copy) [151]. Because the direct repeats are often short, the desired recombination event may be very rare. To select against the integrated plasmid, bacteria are grown in the selective media supplemented with 5 % sucrose [151].

2.7.6.2 DNA digestion and ligation

The target DNA fragments were digested with restriction endonucleases *Sal*I and *Sma*I (or *Sph*I) (New England Biolabs, UK) and ligated to similarly digested pJCB12 using the Quick ligation kit (New England Biolabs, UK) following the manufacture's instruction. The resulting plasmid was transformed into the target cells.

2.7.6.3 DNA plasmid isolation

A single colony was inoculated in 5 mL LB medium containing 15 μg / mL chloramphenicol for 8 hours at 37 °C with shaking at 300 rpm. Two hundred microliters of the starter culture was inoculated in 100 mL of selective medium at 37 °C for 12 - 16 with shaking at 300 rpm. The cell density was approximately 3-4 x 10^9 cells / mL. DNA plasmid was extracted using Qiagen plasmid maxi kits following the manufacturer's instruction (Qiagen, USA). In order to gain pure DNA, the DNA was extra-precipitated in 80 μl 3M NaOAc pH 5 and 500 μl isopropanol and washed twice in 70 % ethanol. Finally, the DNA pellet was dissolved in 50 μl TE which was a solution of 5 μl Tris 0.1EDTA pH8 and 45 μl H_2O.

2.7.6.4 Competent cell preparation

Competent cells are cells that can uptake foreign DNA during electrotransformation, the method used here was adapted from Bjorkman *et al.* with modification [152].

A colony of bacteria was grown in 8 mL of LB overnight. Then, the broth was transferred to a 400 mL prewarmed-LB flask and incubated at 37 °C for 3 hours (or when OD_{600} = 0.3 - 0.4 and < 0.6). The culture was kept on ice and aliquoted into 8 falcon 50 mL tubes- 50 mL / tube. Cells were harvested by centrifugation at 4,000 rpm for 10 minutes which were subsequently re-suspended in 25 mL Elga water for each

tube. Two tubes were combined into one. The cells were centrifuged at 7,000 rpm for 10 minutes. Latterly, cells were then resuspended in 25 mL Elga water / tube. Tubes were combined into 4 tubes (37.5 mL / tube). The cells were resuspended in 25 mL Elga water / tube. Finally, all tubes were combined together. The cells were harvested and resuspended in 800 µl Elga water and supplemented with 15 % glycerol. Competent cells were store in – 80 °C until use.

2.7.6.5 Electro-transformation

This method was adapted from Dower et al. [153] with modifications. Competent cells were thawed at room temperature, and placed on ice. Forty microliters of cells were transferred to a cold 1.5 µl eppendorf; 1 to 2 µl of DNA solution was added to give a final concentration of 7.5 µg / mL (from 10 pg / mL). The suspension was mixed and transferred to a chilled 1 mm electroporation cuvette (Invitrogen, USA). The pulsing was carried out with an electroporator (Bio-rad) which was set to 1.8 kV, 25 µF and 200 Ω. After the electrical pulse, the cells were immediately re-suspended in 1 mL fresh S.O.C solution (Invitrogen, USA) (supplemented with aromix when using BRD 948), transferred to the 20 mL LB tube and incubated at 37 °C in 35 minutes. The transformants were screened by plating 50-100 µl on the LB supplemented with 15 µl / mL chloramphenicol. Mutations were confirmed by sequencing before susceptibility testing was performed for the mutants [153].

2.8 Genotyping methods

A subset of *S.* Typhi isolates which were selected for genotypic investigation is represented in Table 2.5.

Table 2.5 List of *S.* Typhi strains selected for genotyping

Seventy three *S.* Typhi strains from seven Asian countries were selected for SNP typing by GoldenGate assay. Sixteen *S.* Typhi strains which were kindly provided by the Sanger Institute and two Vietnamese strains (CT18 and AG 3) along with an Indonesian strain (J 185SM) were used as haplotype controls. (*) Gifts from the Sanger Institute, Cambridge, United Kingdom.

No	Strain identification	Country	Year	Origin
1	AS18977	Bangladesh	2003	Clinical Isolate
2	AS31279	Bangladesh	2003	Clinical Isolate
3	AS32308	Bangladesh	2003	Clinical Isolate
4	AS-3808	Bangladesh	2003	Clinical Isolate
5	AS-9242	Bangladesh	2003	Clinical Isolate
6	Doni C26	China	2002	Clinical Isolate
7	Doni C36	China	2002	Clinical Isolate
8	Doni C4	China	2002	Clinical Isolate
9	Doni C42	China	2002	Clinical Isolate
10	DoniC51	China	2002	Clinical Isolate
11	B116	India	2003	Clinical Isolate
12	D15	India	2003	Clinical Isolate
13	D186	India	2003	Clinical Isolate
14	D43	India	2003	Clinical Isolate
15	E48	India	2003	Clinical Isolate
16	J-2018XM	Indonesia	2002	Clinical Isolate
17	J-359BM	Indonesia	2002	Clinical Isolate
18	J-80YM	Indonesia	2002	Clinical Isolate
19	L250	Laos	2003	Clinical Isolate
20	L380	Laos	2003	Clinical Isolate
21	L54	Laos	2003	Clinical Isolate
22	L599	Laos	2003	Clinical Isolate
23	BL16599	Pakistan	2002	Clinical Isolate
24	BL21095	Pakistan	2002	Clinical Isolate
25	BL2513	Pakistan	2002	Clinical Isolate
26	BL3723	Pakistan	2002	Clinical Isolate
27	BL820	Pakistan	2002	Clinical Isolate
28	113	Viet Nam	1994	Clinical Isolate
29	255	Viet Nam	1999	Clinical Isolate
30	304	Viet Nam	1998	Clinical Isolate
31	318	Viet Nam	1999	Clinical Isolate
32	436	Viet Nam	2001	Clinical Isolate

No	Strain identification	Country	Year	Origin
33	1592	Viet Nam	1999	Clinical Isolate
34	1697	Viet Nam	2000	Clinical Isolate
35	1727	Viet Nam	2001	Clinical Isolate
36	2017	Viet Nam	2001	Clinical Isolate
37	2062	Viet Nam	2000	Clinical Isolate
38	2694	Viet Nam	1995	Clinical Isolate
39	3069	Viet Nam	2000	Clinical Isolate
40	4313	Viet Nam	1995	Clinical Isolate
41	5623	Viet Nam	1995	Clinical Isolate
42	7560	Viet Nam	1996	Clinical Isolate
43	8071	Viet Nam	1996	Clinical Isolate
44	8699	Viet Nam	1996	Clinical Isolate
45	8951	Viet Nam	1996	Clinical Isolate
46	9019	Viet Nam	1994	Clinical Isolate
47	9183	Viet Nam	1996	Clinical Isolate
48	10158	Viet Nam	1994	Clinical Isolate
49	10877	Viet Nam	1996	Clinical Isolate
50	10880	Viet Nam	1996	Clinical Isolate
51	14308	Viet Nam	1997	Clinical Isolate
52	14635	Viet Nam	1997	Clinical Isolate
53	AG 112	Viet Nam	2004	Clinical Isolate
54	AG 152	Viet Nam	2005	Clinical Isolate
55	AG 165	Viet Nam	2005	Clinical Isolate
56	AG 169	Viet Nam	2005	Clinical Isolate
57	AG 175	Viet Nam	2005	Clinical Isolate
58	AG 25	Viet Nam	2003	Clinical Isolate
59	AG 26	Viet Nam	2005	Clinical Isolate
60	AG 33	Viet Nam	2003	Clinical Isolate
61	AG 336	Viet Nam	2004	Clinical Isolate
62	AG 45	Viet Nam	2004	Clinical Isolate
63	AG 53	Viet Nam	2004	Clinical Isolate
64	DT 2	Viet Nam	2002	Clinical Isolate
65	DT 47	Viet Nam	2002	Clinical Isolate
66	DTY1_94	Viet Nam	1997	Clinical Isolate
67	HTD798	Viet Nam	2003	Clinical Isolate
68	HTD810	Viet Nam	2003	Clinical Isolate
69	Ly Van Tan	Viet Nam	2006	Clinical Isolate
70	Ty.05909	Viet Nam	2004	Clinical Isolate
71	UI-5106	Viet Nam	2004	Clinical Isolate
72	V.04844	Viet Nam	2004	Clinical Isolate
73	V.07641	Viet Nam	2004	Clinical Isolate
74	E98-2068	Bangladesh	1998	Control*
75	E98-3139	Mexico	1998	Control*
76	AG3	Viet Nam	2004	Control
77	8(04)-N	Viet Nam	2004	Control*
78	E02-2759	India	2002	Control*
79	E03-9804	India	2003	Control*

No	Strain identification	Country	Year	Origin
80	ISP-03-07467	Morocco	2003	Control*
81	ISP-04-06979	Central Africa	2004	Control*
82	CT18	Viet Nam	1993	Control
83	E03-4983	Indonesia	2003	Control*
84	404ty	Indonesia	1983	Control*
85	E00-7866	Morocco	2000	Control*
86	E02-1180	India	2002	Control*
87	E98-0664	Kenya	1998	Control*
88	J185SM	Indonesia	1985	Control
89	M223	Unknown	1939	Control*
90	150(98)S	Viet Nam	1998	Control*
91	Ty2	Russia	1916	Control*
92	E0.-6750	Senegal	2001	Control*

2.8.1 Single Nucleotide Polymorphisms (SNP) typing

The high throughput GoldenGate method was applied for analyzing single nucleotide polymorphism (SNP) of *S.* Typhi isolates in this study. This method allows multiplex detection of between 96 and 1,536 nucleotide changes. The procedure of the GoldenGate assay includes 9 steps. Firstly DNA is activated and then is combined with oligonucletides, hybridization buffer and paramagnetic particles. Three primers (oligos) are designed for each SNP locus. Two primers are specific to each allele of the SNP site, called the Allele - Specific Oligos (ASOs). A third primer that hybridizes several bases downstream from the SNP site is the Locus - Specific Oligo (LSO). All three primer sequences contain regions of genomic complementarities and universal PCR primer sites; the LSO also contains a unique address sequence that targets a particular bead type. Secondly, the excess and mis - hybridized primers are removed via several stringency washes. Step 3 is the extension of the appropriate ASO and ligation of the extended product to the LSO; this combines information about each SNP site to the address sequence on the LSO. The full length product from these jointed products provides a template for PCR using universal PCR primers P1, P2 and P3. The universal primers P1 and P2 are labeled with Cy3 and Cy5 dyes. After binding the PCR product, eluting dye - labeled strand, the single - stranded dye - labeled DNAs are hybridized to their complement bead type through their unique address sequences. The assay products are separated onto a solid surface for individual SNP genotype readout. After hybridization, the beadArray Reader is used to analyze fluorescence signal on the Sentrix Array Matrix or BeadChip (8), which is in turn analyzed using software for automated genotype clustering and calling (9) (www.Illumina.com) [154] (Figure 2. 6).

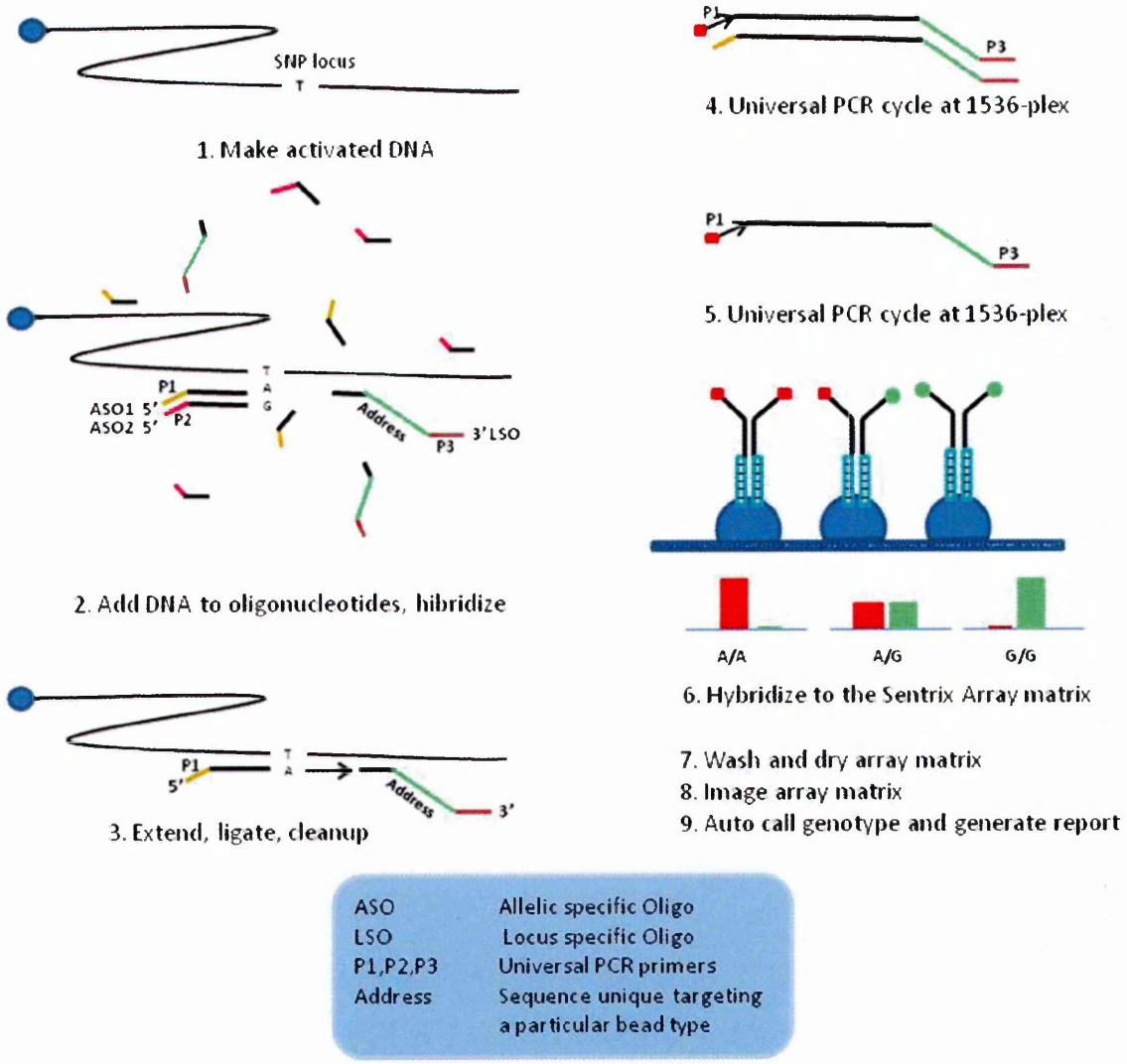


Figure 2.6 The principles of the GoldenGate SNP detection assay methodology

See text for description of protocol.

In this study, 1,508 loci including chromosomal SNPs, chromosomal deletions, *IncHI1* plasmid SNPs, other plasmid genes, and resistance genes were designated in a platform. The SNPs were designed based on previous study [69]. A subset of seventy three Asian *S.* Typhi strains selected for genotyping by the GoldenGate assay were represented in Table 2. 5. In brief, 30 µl of a 10 ng / µl DNA template was made up and duplicated in two plates. AG3 and J185SM DNA templates which were whole genome sequenced were used as SNP typing controls; water was used as a negative control. The DNA samples were arrayed randomly on the plate. The plates of *S.* Typhi DNA were

processed and analyzed using the GoldenGate system at the Sanger Institute, Cambridge, UK following manufacturer's recommendations (www.Illumina.com).

2.8.2 Multiplex Ligation Dependent Probes Amplification (MLPA)

MLPA is a simple and robust multiplex assay that has been applied to observe antimicrobial resistance mutations previously [155]. The specificity and sensitivity of the assay were ensured at the initial ligation step and PCR amplification. The sizes of the MLPA product correspond to the targeted SNPs. This assay offers great advantages in which multiple mutations distributed throughout the genome can be targeted in a single assay without compromising the sensitivity, specificity, or simplicity of the method [155]. Generally, the target sequence was identified by hybridization of two adjacent complementary probes. The probes are subsequently joined by a ligation reaction, and copy sequences are amplified in a multiplex PCR reaction using unique primers attached to the probes (Figure 2.7). Only probes hybridized to the target sequence will be ligated and subsequently amplified in the PCR reaction. After separation by capillary gel electrophoresis, the amplification product can be analyzed [156].

2.8.2.1 Design of probes

Sixteen discriminatory markers were selected and MLPA probes were designed accordingly following manufacturer's recommendation [157]. These markers including 11 insertion - deletion regions which were haplotype representative published previously [69,71], two indicators of *gyrA* and *parC* mutations conferring nalidixic acid resistance and reduced susceptibility to fluoroquinolone and three species controls including *aroC, ssaV* and *fliC* genes. These genes are vitally responsible for the *S.* Typhi metabolism. Wherein, the *aroC* gene encodes chorismate synthase, an enzyme

involved in the biosynthesis of aromatic compounds [158]. The *ssaV* gene encodes a component of the type III secretion system located on *Salmonella* pathogenicity island 2 (SPI-2) which is required for *Salmonella enterica* survival and growth within macrophages [159]. Finally, the *fliC* gene encodes bacterial flagella [160]. The probe sequences are represented in Table 2. 6.

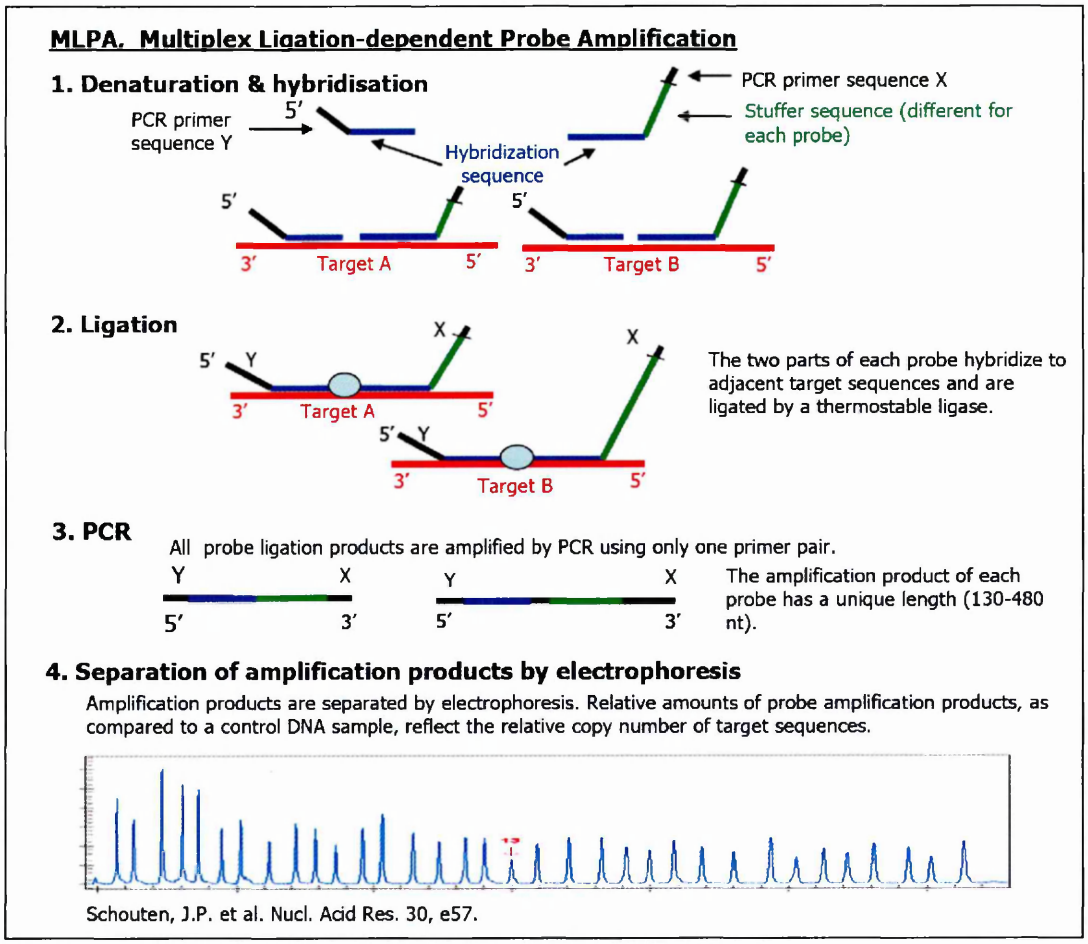


Figure 2.7 MLPA-Multiplex PCR Ligation-dependent probe amplification

The figure was adapted from Schouten (2002) [157].

Table 2.6 List of probes for *S.* Typhi strain genotyping by the MLPA method

Nine fragments with lengths of 68 bp, 74 bp, 80 bp, 87 bp, 94 bp, 99 bp, 104 bp, 109 bp, 114 bp were used as control fragments and were from human genetic sources and are not included in the table. The underlined red letters are the nucleotides in which the mutations in *gyrA* and *parC* genes in *S.* Typhi strain were targeted. The length represents the length of each insertion and deletion fragments. The names are the name of the specific insertion and deletion fragments [69]. These insertion and deletion fragments include the PARC54 and GYR8387 probes, which were designed for the detection of mutations in *gyrA* and *parC* genes of *S.* Typhi. The spanning oligo was specially designed to detect the mutations at position 83 and 87 of the *gyrA* gene of *S.* Typhi ; The B, Q, A, K, D, E, F, S, C, H, N and D are the insertion and deletion fragments; the AROC1, FLI1 and SSAV1 are the internal positive controls for *S.* Typhi.

Length (bp)	Name	Left hybridization sequence, LHS	Spanning oligo	Right hybridization sequence, RHS
130	PARC54	TCAATACGACTCTAGATTAGACACCATGGCTTCATAGC AGGCGA		TGTCGCCATGCGGGTGATACTTACCTATTATGCTGAGC
139	B	TTCCGGTCAGACTTGGACGCAAAATACAGGTA		CGAAAGGCCAGCTCGTGAAAATCCATAGAGGGGCT
158	Q	TTATGGACGCTGGTGCAGCAGGAG		CCCTCCCATCCGTTAATTGTTAGTACCACGCTGAACACCG
175	A	TTTTAGTCCATCACGCGCTTCAACATCCGTTC		TATCAGGATGTTGAAAAAGTGGTGCGCATTAATCCACTGAACAC
204	K	TCGACTGGCTGGACATTTTGTGGTTC		TCTGCCGATGCGCTGGAGCAGCTTTGTGATGCGCT
238	GYR8387	CCAGCGAGAATGGCTGCGCCATACGAACGA	TGGTGT CATACA CTGCGA	AATCGCCGTGGGGATGGTATTTACCGATTACGTCACCAACGAC
252	E	GGAACATGGCGATATCAAATCCATCAATG		AAAGCGTCCAGTCCCTGTTTGATGCTCAGTCCCTG
267	AROC1	GACAACAACGATAACGGAGCCGTGATGGCA		GGAAACACAATTGGACAACTCTTTCGCGTAACCACTTTCG
275	S	GCTCTCTGGCTTTTTGAACACAGAAATGG		TCGAGTGAAATCAGCTTTGACTTAGCCCTTTTATAAAGCCTTGCGGC
308	F	GCAGTCAGATTTGAACGTCCGGCATAA		AAATAAGTGCTATTAAGCTCTTCAACTGTATCCATACTCTAATTTCCTG
362	C	CTAAAGCGTCGTTGATAATGGTGTAACGGT		GAACTGTCACAGGTCGTGGCGTTATCTTCGATAAACTTCACCT
374	FLIC1	CTGTTGACCCAGAATAACCTGAACAAATCC		CAGTCCGCACTGGGCACTGCTATCGAGCGTTTGT
414	H	CTTGCTTTACCGAGCAGCATATTCTTTCCGCT		ATGACCGTTCAGTATTTGCAGAAGAAAGGCTTTCAGGTTCAGCC
429	SSAV1	CCTGATTCCCTCACCTAACCATGAAC		GCATTGCGACTCCAGAAATTTTATTTGTCGATGATGTAATCGTAACC
465	N	CCAGCACAGGCGTATTATCGCTAACACGCT		CACCAACATAGTCGATTTCATGCCAGTAAACTATTGGTAGCCTGGT
494	D	CCTGGTCAAGCGTTAACTTTATTACTGCCCAT		GACGGCTTCACGCTGAACGATCTGGTGTCTTATAACGA

Among these probes, the *gyrA* probe was optimized to give lower signal if any other variant of wild type (TCC - GAC) is presented. The variants need to be sequenced to verify the exact nucleotide change.

The MLPA reagents and method was kindly provided and instructed by the MRC-Holland, Amsterdam, The Netherlands [157]. The MLPA procedure includes three following steps.

2.8.2.2 DNA hybridization and ligation

Five microliters of DNA samples (100 ng / µl) were heated at 98 °C for 10 minutes in a thermocycler (ICycler, BioRad, USA) with a heated lid. These tubes were subsequently cooled to 25 °C before adding a mixture of 1.5 µl SALSA probemix and 1.5 µl MLPA buffer to each tube. Samples were heated for 1 minute at 95 °C and then incubated for 16 hours at 60 °C. Ligation of annealed oligonucleotides was performed by diluting the sample to 40 µl with dilution buffer containing 3 µl Ligase-65 buffers A, 3 µl ligase-65 buffer B, 1µl ligase - 65 and 25 µl water, and incubated 15 minutes at 54 °C, then heated for 5 minutes at 98 °C.

2.8.2.3 DNA amplification

Forty microliters PCR reaction was performed by adding 10 µl polymerase mix containing 2 µl SALSA Enzyme Dilution buffer, 5.5 µl water, 0.5 µl SALSA Polymerase and 2 µl SALSA PCR-primers which include a forward primer (labeled) (5′ -GGGTTCCCTAAGGGTTGGA - 3′) and a reverse primer (unlabeled) (5′ - GTGCCAGCAAGATCCAATCTAGA - 3′) into 30 µl PCR buffer mix while at 60 °C. In which, the PCR buffer mix was made up in advance by suspended 4 µl SALSA PCR buffer and 26 µl water into 10 µl of the ligation reaction. A thirty five-cycle PCR

programme was performed and included 30 s at 95 °C, 90s at 65 °C, and 60 s at 60 °C, and following by 72 °C for 20 minutes.

2.9 Analysis methods

2.9.1 Fragment analysis

MLPA products were either simply visualized using the agarose gel electrophoresis (Section 2.8.3) or the capillary sequence system ABI 3130XL with fragment analysis software (Genemapper V4.0 ABI Applied Biosystems, USA). A half microliter of PCR product which was amplified with one unlabelled and one FAM - labeled primer was mixed with 9.25 µl Hi-di Formamide and 0.25 µl LIZ - 500 size standard (GeneScan, ABI Applied Biosystems, USA). The mixture was incubated in 3 minutes at 95 °C, then immediately chilled on ice for few minutes before analyzed on a 3130XL ABI capillary electrophoresis system.

2.9.2 MLPA analysis

Generally, the combination of 16 peaks appeared with the length varied from 130 bp to 494 bp along with other 9 control fragments covering the region 68 bp - 114 bp which were the manufacturer's controls. Among these 9 control peaks, four fragments of 68 bp, 74 bp, 80 bp and 87 bp were designed to give a signal if too low input DNA was used. These peaks gave similar heights. If an appropriate amount of DNA was used in the experiment, these peaks rose to half or less than half of the general peak heights of the *S.* Typhi probes.

2.9.3 Electrophoresis

Agarose gels (0.8 - 3 %) (Biorad, USA) were used to visualize DNA amplifications. Different agarose concentrations were used depending on the purpose of each

experiment. Agarose gels were stained with 0.3 mg / mL ethidium bromide and visualized under UV light and examined using the Gel Doc system (Biorad, USA), bands were interpreted using Quantity One software (Biorad, USA).

2.9.4 Bioinformatics analysis

The DNA sequences that were selected for further analysis were searched via the website of the National Centre for Biotechnology Information (NCBI) (http://ncbi.nlm.nih.gov/BLAST) usin g the BLAST alignment tools. DNA sequence alignment was conducted using AlignX programme of the VectorNTI Suit 7 software.

2.10 Statistics and mathematical methods

2.10.1 Statistical analysis for the competition assay

For each competition experiment, the mean of 6 readings in two plates was used for the calculation of the relative competitive fitness. The CFU counts on nalidixic acid agar indicate the number of nalidixic acid resistant cells in the mixed cultures. The number of susceptible cells was calculated by subtracting the number of resistant cells from the total cell number revealed by the CFU counts on the plates that did not contain antimicrobials.

2.10.2 Statistical method for other calculations

Means, medians, drug resistance sample correlations and graphs of standard statistical distributions were analyzed using R commander software v2.9.0 [161].

3. Characterization of *Salmonella* Typhi antimicrobial susceptibility patterns in Viet Nam and other Asian countries

3.1 Introduction

There are approximately 21 million cases of typhoid fever worldwide per year with the majority of those cases occurring in Asia [29]. An estimated 220,000 deaths per year occur as a consequence of typhoid fever [29]. Since the isolation of multidrug resistant (MDR) *S.* Typhi, which shows resistance to all first-line antimicrobials (chloramphenicol, ampicillin and sulfamethoxazole-trimethoprim) in the 1980s, the fluoroquinolone class of antimicrobial has become the preferred treatment for enteric fever [15,19]. Outbreaks of typhoid caused by *S.* Typhi strains that were resistant to nalidixic acid (the prototype quinolone, which is used for *in vitro* screening testing) have been reported in a number of countries [9]. Whilst nalidixic acid is not used therapeutically for treatment of typhoid, these strains also have reduced susceptibility to fluoroquinolones. Viet Nam, particularly the Mekong Delta region of southern Viet Nam, has faced a series of typhoid fever epidemics over the last decade [38,162-164]. These epidemics were imposed on a background of endemic disease, however, these outbreaks may have reflected a change in resistance patterns of the organisms and we hypothesized that antimicrobial resistance was becoming a serious problem in hampering effective typhoid treatment in southern Viet Nam [165].

In order to investigate the magnitude of antimicrobial resistance and the patterns of drug sensitivity in southern Viet Nam we amassed a collection of *S.* Typhi isolates that were representative of this location and would offer a snap-shot of any antimicrobial resistance transition occurring over this period of time. In total, the collection that was analyzed was comprised of 1,388 *S.* Typhi isolates. These *S.* Typhi isolates were collected from 18 southern provinces of Viet Nam between 1993 and 2005. The

provinces that these strains were collected from are outlined in Table 1 and the provincial locations are pictorially represented in Figure 3.1.

Figure 3.1 The map of provinces in southern Viet Nam where *S.* Typhi strains were collected

The provinces where *S.* Typhi isolates were collected were An Giang, Ba Ria, Ben Tre, Binh Thuan, Dak Lak, Dong Nai, Ho Chi Minh, Kien Giang, Long An, Soc Trang, Song Be, Tay Ninh, Thuan Hai, Tien Giang, Tra Vinh, Phu Dong and Vinh Long.

Typhoid fever is endemic and a public health issue in Viet Nam and in other Asian countries in the vicinity. Whilst the collection of strains that was representative of those in Viet Nam, are important to understand the national situation, it is essential to relate this information to the antimicrobial resistance patterns in other locations. The movement of people in South East Asia is significant; therefore, the dissemination of any antimicrobial resistant *S.* Typhi may occur at high frequency.

To compare the national situation to the overall Asian situation we generated a cross sectional sample collection of *S.* Typhi isolates from eight Asian countries. Many of these strains were collected as part of a multi-centre, multi-faceted study of typhoid in Asia. This study formed part of the International Vaccine Institute's (IVI) Diseases of the Most Impoverished (DOMI) investigation [129]. The DOMI project sought to calculate the burden of typhoid fever and other diseases related to poverty in southern Asia. Our laboratory directed the sample collection and description of the strains circulating in Asia during this period of investigation. The *S.* Typhi strains were collected over a five year period (2002 – 2006) in eight Asian countries where typhoid is known to be endemic. These countries are highlighted in Figure 3.2, and were Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan and some additional strains from central Viet Nam. Typhoid is endemic in all the countries described and these nations are home to more than 80 % of the world's typhoid fever cases [29] (Figure 3.2).

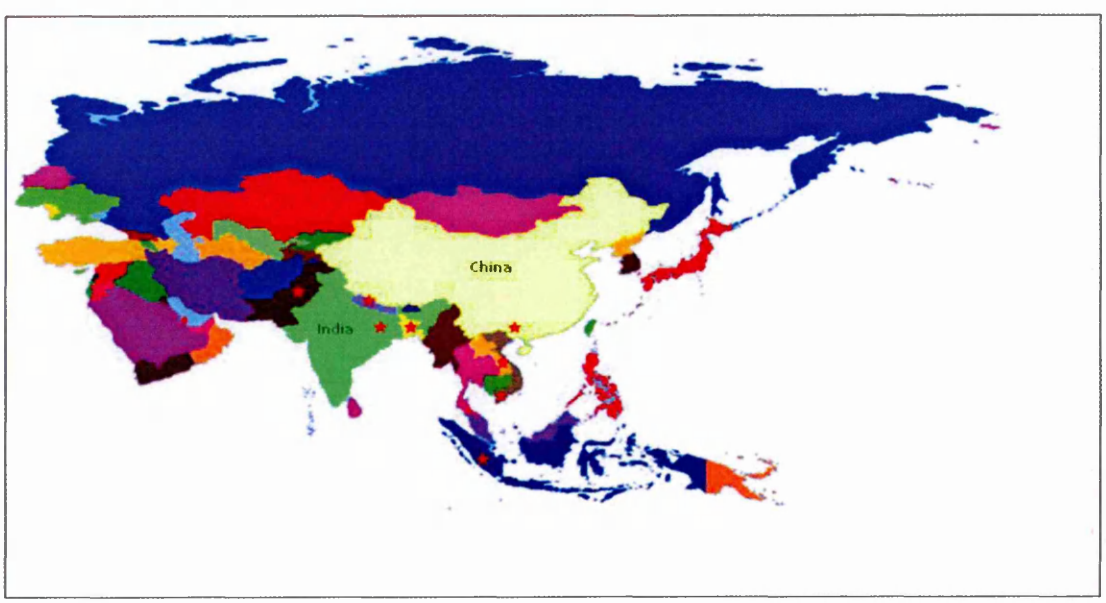


Figure 3.2 The map of Asian countries involved in this study

(★) Red stars represent the locations of the countries where *S.* Typhi were collected. The countries were Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan and Viet Nam.

The emergence and spread of antimicrobial resistant *S.* Typhi means limited treatment options for typhoid fever in many countries where the disease is endemic. The information of extension and patterns of drug resistant *S.* Typhi across Asia is vital to guide treatment policy and is also important for governments planning potential vaccination campaigns. Areas with high transmission and high levels of antimicrobial resistant strains should be a potential target for intervention strategies. Here we compare and contrast the antimicrobial resistance profile in the *S.* Typhi population in Viet Nam and in other locations in the South of Asia. This collection of 2,028 strains represents the largest number of *S.* Typhi isolates investigated in this manner a nd permits a snapshot view of the situation of antimicrobial resistance in these locations.

3.2 Results

3.2.1 *S.* Typhi collection

3.2.1.1 Vietnamese *S.* Typhi strain collection

Of the 1,388 strains constituting the Vietnamese *S.* Typhi collection used for analysis, 85 % of strains were collected in three locations in southern Viet Nam. These three locations represent areas of high incidence and were An Giang province, Dong Thap province and Ho Chi Minh City. Five hundred and twenty three strains, 366 strains and 323 strains were isolated from Dong Thap province, An Giang province and Ho Chi Minh City respectively. The strains were collected over a 13 year period. However, 1994 (214 strains), 1995 (256 strains) and 2004 (201 cases) were the 3 years with the highest number of organisms isolated (Table 3.1).

Table 3.1 The distribution of *S.* Typhi isolates which were collected in the provinces of southern Viet Nam between 1993 and 2005

Year / Province	1993 (N=61)	1994 (214)	1995 (256)	1996 (67)	1997 (134)	1998 (118)	1999 (94)	2000 (36)	2001 (17)	2002 (99)	2003 (21)	2004 (201)	2005 (67)
An Giang	2	13	6		5	2	1		1		21	201	67
Ba Ria					1	1							
Ben Tre		1	2		1	1							
Binh Thuan	1												
Dak Lak	1												
Dong Nai	1	3	63	51		2	1						
Dong Thap	2	74	134		32	43	89	37	15	99			
Ho Chi Minh	27	90	47	12	82	63	2						
Kien Giang						1	1						
Long An	6	6		1	5								
Soc Trang			1		1								
Song Be		1	1	1									
Tay Ninh		5											
Thuan Hai			1										
Tien Giang	18	18	1	1	6	4							
Tra Vinh	2	1											
Vinh Long	1	2		1									
Phu Dong						1							

3.2.1.2 Asian strain collection

One hundred and forty-nine *S.* Typhi isolates were collected in March and April 2003 during a hospital-based descriptive study at Patan Hospital, Kathmandu, Nepal. Fifty isolates were collected consecutively during a clinical trial in 2002 and 2003 at the Wellcome Trust-Mahosot Hospital, in the Lao People's Democratic Republic [128]. Forty isolates were collected between February and December 2003 in an urban slum in Dhaka, Bangladesh [130]. Additionally, 21 isolates were collected during 2002 in Hechi city, Guang Xi, China; 23 strains were collected from May to July 2003 in slum areas in Kolkata, West Bengal, India and 259 strains were collected across India. Seventeen isolates were collected from July to September 2002 in North Jakarta, Indonesia. Thirty four strains were isolated between January 2002 and March 2003 in one slum area in Karachi, Pakistan and 47 isolates were collected between July 2002 and September 2004 in Hue city, central Viet Nam.

3.2.2 Identification

Two thousand six hundred and twenty seven (2,627) strains previously identified in other laboratories as *S.* Typhi were re-cultured and we sought to confirm accurate identification. Microbiological and biochemical identification methodology were performed as described in section 2.2. Of all the strains that constituted the *S.* Typhi strain collection amassed for this study, we confirmed accurate biochemical identification (using short set or API 20E) on 2,028 strains (Table 3.2). The additional strains were confirmed to be either *S.* paratyphi A strains or other *Salmonella* spp.

Table 3.2 Short biochemical characteristics of *S.* Typhi in Asian countries (2002 - 2006) and Viet Nam (1993 - 2005)

(+); positive testing, (-); negative testing. See Section 2.2.2 for technical description. na; not applicable

Country	Strain (N=2627)	KIA	H2S	Gas	Motile	Indol	Urea	Citrate	Methyl red
Bangladesh	40/49	Alk/A	+	-	+	-	-	-	+
China	21/53	Alk/A	+	-	+	-	-	-	+
India_2003	23/27	Alk/A	+	-	+	-	-	-	+
India_2006	259/417	Alk/A	+	-	+	-	-	-	+
Indonesia	17/28	Alk/A	+	-	+	-	-	-	+
Pakistan	34/54	Alk/A	+	-	+	-	-	-	+
Laos, Mahosot Hospital	50/58	Alk/A	+	-	+	-	-	-	+
Nepal- Patan hospital	149/205	na	na	na	na	na	na	na	na
Central Viet Nam	47/49	Alk/A	+	-	+	-	-	-	+
Southern Viet Nam	1388	Alk/A	+	-	+	-	-	-	+

All isolates subjected for further investigations were confirmed by serological testing. This was performed according to WHO guidelines (2003) as described in section 2.2.1. Serological identification results are presented in Table 3.3. All *S.* Typhi isolates defined by short biochemical set or API20E tests were positive to polyO and O_9 agglutination reactions, regardless of the Vi antigen reaction. In total 68 % (572 / 841) of all tested isolates were confirmed as *S.* Typhi using serological testing. The Vi antigen is known to be variable during culture and some *S.* Typhi strains have been found in Pakistan that are Vi Negative and incapable of expressing the Vi antigen [166]. Our data demonstrates that 93 % (531 / 572) of the isolates tested had positive agglutination when tested for expression of the Vi antigen, 6 % (37 / 572) were negative for agglutination and 1 % (4 / 572) gave un-interpretable results.

Table 3.3 Serological identification of *S.* Typhi strains isolated in Asia (2002 -2006)

(+); agglutination, (-); no agglutination. polyO, polyvalent O antigen, the somatic antigen specified *Salmonella* species. O_9, somatic antigen specified *S.* Typhi. Vi+; providing agglutination to virulent antigen, Vi-; not providing agglutination to Vi antigen, Vi-/+, not providing clear agglutination to Vi antigen.

Country	Strains (N=841)	polyO	O_9	Vi +	Vi -	Vi-/+
Bangladesh	40/49	+	+	40		
China	21/53	+	+	21		
India (2003)	23/27	+	+	19	4	
India (2006)	259/417	+	+	218	7	4
Indonesia	17/28	+	+	17		
Pakistan	34/54	+	+	34		
Laos	50/58	+	+	50		
Nepal	149/205	+	+	59	15	
Central Viet Nam	47/49	+	+	36	11	
Southern Viet Nam	201/326	+	+	37		

3.2.3 Characterization of antimicrobial susceptibility

Two thousand and twenty eight isolates (including 1,388 isolates from Viet Nam and 640 isolates from other Asian countries) which were confirmed *S.* Typhi were analyzed using MIC susceptibility testing (Figure 3.3). Five non - quinolone and four quinolone / fluoroquinolone antimicrobial agents were used for susceptibility testing. The five non - quinolone antimicrobials tested were ampicillin, chloramphenicol, sulfamethoxazole - trimethoprim (co - trimoxazole), ceftriaxone and azithromycin. The quinolone / fluoroquinolones tested were nalidixic acid, ofloxacin, ciprofloxacin and gatifloxacin. MIC values were interpreted according to CLSI guidelines in which the MIC breakpoints for the antimicrobial agents described in section 2.4.

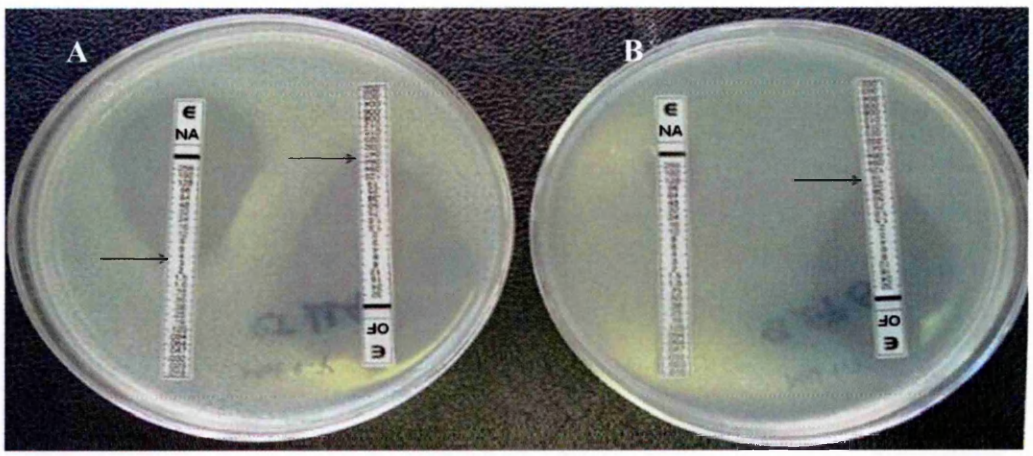


Figure 3.3 Representative MIC E - tests for examining fluoroquinolone susceptibilities of *S.* Typhi strains

A, *S.* Typhi isolate CT 117 from Viet Nam, demonstrating sensitivity to nalidixic acid (NA) and ofloxacin (OF). The MIC of NAL (2 µg / mL) and OF (0.03 µg / mL) to this organism were read according to the manufacturers recommendation and are distinguished by arrows.

B, *S.* Typhi isolate 9180 from Viet Nam, demonstrating resistance to nalidixic acid (NA) and sensitivity to ofloxacin (OF). The MIC of NA (256 µg / mL) and OF (0.25 µg / mL) to this organism were read according to the manufacturers recommendation and are distinguished by arrows.

3.2.3.1 Multidrug resistance in Asian countries from 2002 to 2006

The antimicrobial susceptibilities of 841 *S.* Typhi strains isolated between 2002 to 2006 from eight Asian countries were analyzed to the five non - quinolone antimicrobials to assess MDR rates (Table 3.4). MDR *S.* Typhi is defined as an organism that exhibits resistance to chloramphenicol, ampicillin and co - trimoxazole. MDR *S.* Typhi originated in the 1990s and is related to the organisms carrying an MDR plasmid.

There were variable rates of MDR across the seven sites. Over the collection period, there were two independent collections arising from India, in 2003 and 2006 (Figure 3.4). The MDR rate in the Indian collections decreased over a period of three years, although the initial collection in 2003 only contained 23 strains. In locations where an MDR phenotype was found, the lowest was in the previously mentioned Indian collection from 2006 where 10 % (27 / 259) were MDR, whilst the highest was 45 % (90 / 201) from southern Viet Nam (Figure 3.4). Somewhat surprisingly, both China and Indonesia were exceptions with no MDR *S.* Typhi isolates identified.

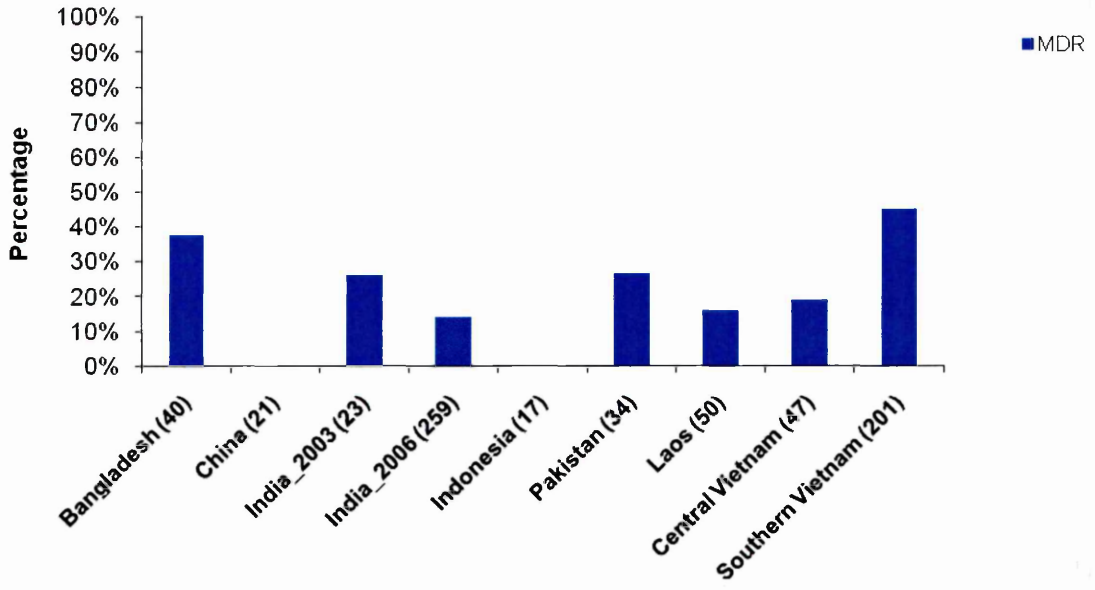


Figure 3.4 Multidrug resistant *S.* Typhi isolates from seven Asian countries (2002 – 2006)

The blue bars indicate the percentage of multidrug resistant *S.* Typhi isolates which were collected from seven Asian countries including Bangladesh, China, India (2003 and 2006), Indonesia, Pakistan, Laos, central and southern Viet Nam. The number in brackets is the number of *S.* Typhi isolates in each country, the total is 841 isolates.

Table 3.4 Antimicrobial susceptibility of *S.* Typhi isolates in Asian countries between 2002 and 2006

CHLR; chloramphenicol resistance, AMPR; ampicillin resistance, SXTR; sulfamethosazone-trimethoprim resistance, CRO range; the MIC range of ceftriaxone (the minimum and maximum MIC values (μg / mL) of ceftriaxone to *S.* Typhi isolates are represented), CRO; ceftriaxone, AZMR; Azithromycin resistance, MDR; multidrug resistance (*S.* Typhi isolates resistant to ampicillin, chloramphenicol and sulfamethosazone-trimethoprim). The MIC breakpoints according to CLSI guideline of each antimicrobial used in this study are depicted on the header, under the antimicrobial name. The percentage along with the number of resistant *S.* Typhi strains per total strains in brackets are depicted in the columns of CHLR, AMPR, SXTR, AZMR and MDR. $MIC_{50/90}$, the MIC values where 50% and 90% of *S.* Typhi were inhibited, respectively. na; not applicable.

Country	CHLR (<8 and >32)	AMPR (<2 and >8)	SXTR (<2/38 and >8/152)	CRO range (<8 and >64)	CRO ($MIC_{50/90}$)	AZMR (<2 and >8)	MDR
China	0% (0/21)	0%	0%	0.047-0.19	0.094/0.125	14% (3/21)	0% (0/21)
Indonesia	0% (0/17)	0%	0%	0.047-0.125	0.094/0.094	0%	0% (0/17)
Laos	18% (9/50)	20% (10/51)	18% (9/51)	0.015-0.125	0.03/0.06	na	16% (8/50)
Bangladesh	40% (16/40)	100% (40/40)	40% (16/40)	0.06-0.125	0.064/0.125	0%	37.5% (15/40)
India_2003	26% (6/23)	26% (6/23)	26% (6/23)	0.064-0.125	0.125/0.125	4% (1/23)	26% (6/23)
India_2006	14%(35/259)	11% (29/259)	11% (29/259)	na	na	7% (17/259)	10%(27/259)
Nepal	19% (28/149)	na	na	0.002-6	0.094/3	na	na
Pakistan	26.5% (9/34)	17% (9/54)	17% (9/54)	0.064-0.19	0.094/0.125	0%	26.5% (9/34)
Central Viet Nam (IVI)	21.3% (10/47)	19% (9/47)	19% (9/47)	0.032-0.19	0.094/0.19	0%	21.3% (10/47)
Southern Viet Nam (HTD)	46% (92/201)	49% (89/181)	49% (89/181)	0.064-0.19	0.125/0.125	0%	45% (90/201)

Comparing resistance to individual antimicrobials, the percentage of chloramphenicol resistant strain varied from 14 % (35 / 259) in India (2006) to 46 % (92 / 201) in southern Viet Nam (Table 3.4). The percentage of strains resistant to ampicillin ranged from 11 % (29 / 259) in India (2006) to 100 % (40 / 40) in Bangladesh (Table 3.4). The highest percentage of sulfamethoxazole - trimethoprim resistance was observed in southern Viet Nam, 49 % (89 / 181), whilst the lowest percentage of sulfamethoxazole - trimethoprim resistance was in India in 2006 at 11 % (29 / 259).

In the Asian strains examined for an MDR phenotype, there were no ceftriaxone resistant strains identified. Only one *S.* Typhi isolate was found with reduced susceptibility to ceftriaxone (MIC= 6μg / mL), this strain was isolated in Nepal (1/149) (Table 3.4). Fourteen percent (3 / 21) of isolates from China, 7 % (71 / 259) and 4 % (1 / 23) of isolates from India 2006 and 2003 respectively were resistant to azithromycin (There are no CLSI azithromycin MIC breakpoints for *Enterobacteriaceae*, so this result were interpreted using MIC breakpoints of non – enterobactereace [135]), which is one of the current recommendations for the treatment of patients with typhoid caused by MDR *S.* Typhi (Table 3. 4). In a recent randomized trial that evaluated the efficacy of gatifloxacin *versus* azithromycin for the treatment of typhoid fever in southern Vietnam, 58% (58/263) of *S.* Typhi isolates were MDR resistant and 96.5% (254/263) of *S.* Typhi were nalidixic acid resistant. The MIC 50 and MIC 90 (range) of the 263 *S.* Typhi isolates for azithromycin was 8 μg/ml and 12 mg/ml (1.5-16 μg/ml), respectively. In this trial patients showed excellent clinical response to both treatments [161].

3.2.3.2 Multidrug resistance in southern Viet Nam from 1993 to 2005

Between 1993 and 2005, we collected 1,388 isolates of *S.* Typhi from southern Viet Nam. All strains were tested for MDR. The proportion of MDR *S.* Typhi was 67 % (41

/ 61 strains) in 1993 and increased to more than 80 % in the following years. The rate declined in the mid 1990's and was maintained at approximately 90 % at the beginning of the 2000's. The proportion of MDR *S.* Typhi finally increased to 76 % (53 / 70) in 2005, however, this increase was not statistically significant when compared with 1993 onwards ($p = 0.685$, using a Pearson chi-square test with CI 95%). A subset of *S.* Typhi isolates from Dong Thap province of southern Viet Nam (1997-2002) was subjected to analysis to identify any increase in the proportion of MDR over a prolonged period of time (Figure 3. 5). The proportion of MDR strains was consistently at a high level and only varied between 81 % and 97 %. No statitstically significantly difference in MDR percentages from 1997 to 2002 ($p = 0.64$ using Pearson's chi-squared test) was observed.

3.2.3.3 Nalidixic acid resistance and reduced susceptibility to fluoroquinolones in Asian countries

The percentage of nalidixic acid resistant *S.* Typhi demonstrated significant variation when related to the location of isolation (Figure 3.6). No strains from Indonesia or Laos demonstrated resistance to nalidixic acid. Strains from China, Bangladesh and Pakistan had a relatively low incidence of nalidixic acid resistance (< 40 %). The rates of resistance to nalidixic acid in Nepal and India (2006 and 2003) were comparable (≈ 50 %) (Figure 3.6). The highest proportion of nalidixic acid resistant strains in this period (2002 – 2006) was also found in southern Viet Nam (90 %).

All isolates that were resistant to nalidixic acid were tested for resistance and reduced susceptibility to ofloxacin, ciprofloxacin and gatifloxacin. The MIC_{50} and MIC_{90} for these strains were calculated and are presented in Table 3.5. All tested strains, with the exception of two from India (2006) and two from Nepal were sensitive to ofloxacin.

The highest MIC_{90} for ofloxacin was seen in Nepal and southern Viet Nam (1.5 μg / ml). Four percent of strains from Nepal were resistant to ciprofloxacin and all strains tested in all locations were sensitive to gatifloxacin. The highest MIC_{50} and MIC_{90} values of *S.* Typhi to ciprofloxacin were 0.38 μg / mL and 0.5 μg / mL respectively, and those of *S.* Typhi to gatifloxacin were 0.125 μg / mL and 0.25 μg / mL, respectively (Table 3.5). The overall combination of MDR and nalidixic acid resistance was found to be 4.3 % (2 / 47) of *S.* Typhi strains from central Viet Nam, 8.7 % (2 / 23) from India, 23.5 % (8 / 34) from Pakistan and 30 % (12 / 40) from Bangladesh. In Nepal, 18.1 % (27 / 149) of *S.* Typhi were resistant to chloramphenicol and nalidixic acid.

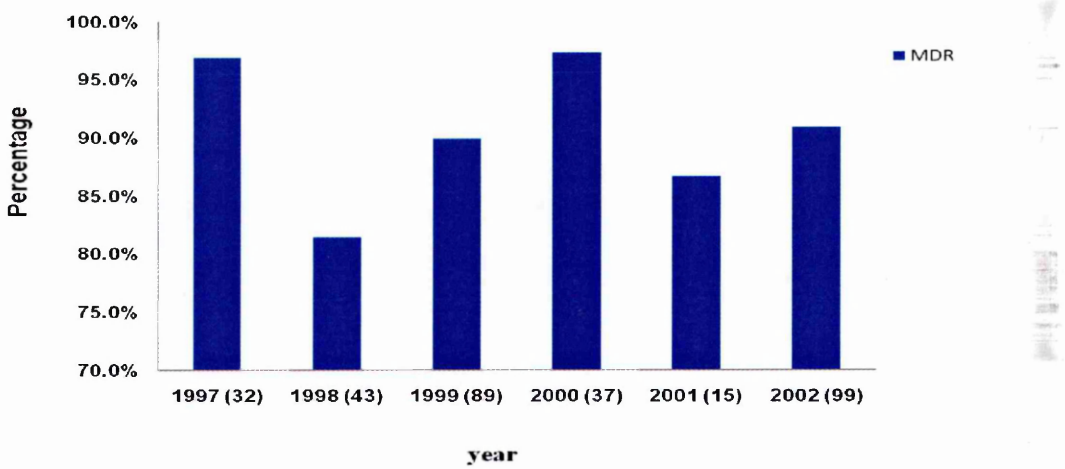


Figure 3.5 Multidrug resistance of *S.* Typhi isolates in Dong Thap province of southern Viet Nam (1997 – 2002)

The blue bars indicate the percentage of multidrug resistant *S.* Typhi isolates which were collected from Dong Thap province of southern Viet Nam from 1997 to 2002. The number in brackets is the number of *S.* Typhi isolates in each year; the total number is 315 isolates. The *p* mean = 0.64 with CI 95% using Pearson's chi-squared test.

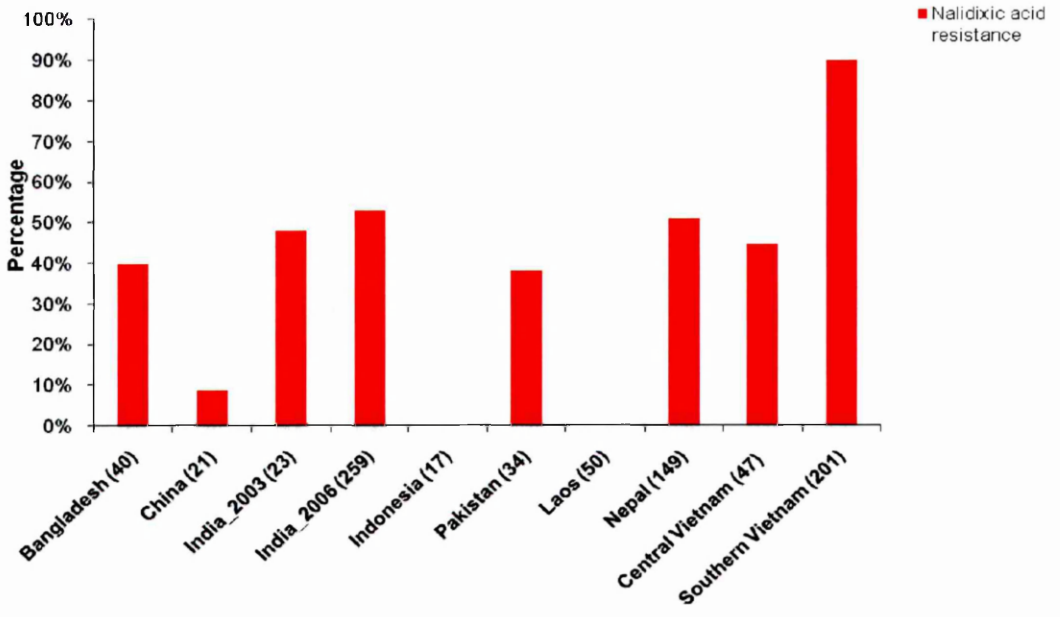


Figure 3.6 Nalidixic acid resistance of *S.* Typhi strains across Asian countries between 2002 and 2006.

Red bars indicate the percentage of nalidixic acid resistant *S.* Typhi isolates which were collected from eight Asian countries including Bangladesh, China India (2003 and 2006), Indonesia, Pakistan, Laos, Nepal central and southern Viet Nam. The number in brackets is the number of *S.* Typhi isolates in each country, the total number is 841 isolates.

Table 3.5 The fluoroquinolone susceptibility of *S.* Typhi isolates across eight Asian countries between 2002 and 2006

MIC_{50}, MIC_{90}; Minimum Inhibitory Concentrations at which 50% and 90% of the organisms respectively are inhibited. Range; the minimum and maximum MIC detected in *S.* Typhi isolates. OFX resistance; Ofloxacin resistance, CIP resistance; ciprofloxacin resistance, NA; not applicable. According to CLSI guidelines, the MIC breakpoints of ofloxacin are ≤ 2 and ≥8, the MIC breakpoints of ciprofloxacin are ≤ 2 and ≥4, and the MIC breakpoints of gatifloxacin are ≤ 2 and ≥8. (*) 2/149 *S.* Typhi strains (**) 6/149 *S.* Typhi strains

Country	ofloxacin (µg/mL)			OFX resistance	ciprofloxacin (µg/mL)			CIP resistance	gatifloxacin (µg/mL)		
	Range	MIC_{50}	MIC_{90}		Range	MIC_{50}	MIC_{90}		Range	MIC_{50}	MIC_{90}
China	0.03-0.5	0.06	0.06	0%	0.008-0.25	0.015	0.03	0%	0.012-0.190	0.023	0.023
Indonesia	0.032-2	0.03	0.03	0%	0.002-0.03	0.015	0.015	0%	0.012-0.023	0.016	0.023
Laos	0.03-1	0.03	0.125	0%	0.006-0.023	0.012	0.016	0%	0.012-0.047	0.016	0.023
Bangladesh	0.003-0.75	0.064	0.75	0%	0.006-0.38	0.025	0.38	0%	0.012-0.19	0.016	0.19
India_2003	0.032-0.5	0.38	0.5	0%	0.006-0.25	0.094	0.25	0%	0.012-0.19	0.125	0.19
India_2006	0.016-12	0.19	0.5	0.5%	0.003-6	0.09	0.25	0.5%	na	na	na
Nepal	0.008-12	0.38	1.5	**1.3%** (*)	0.002-32	0.125	0.5	4% (**)	0.0012-1.5	0.08	0.25
Pakistan	0.023-2	0.047	0.5	0%	0.004-0.25	0.012	0.25	0%	0.012-0.19	0.023	0.19
Central Viet Nam (IVI)	0.03-1	0.023	0.38	0%	0.006-0.5	0.023	0.38	0%	0.008-0.25	0.016	0.19
SouthernViet Nam (HTD)	0.023-2	1	1.5	0%	0.008-0.75	0.38	0.5	0%	0.006-0.25	0.125	0.19

3.2.3.4 Nalidixic acid resistance and reduced susceptibility to fluoroquinolone in southern Viet Nam

From the data of nalidixic acid resistance in southern Viet Nam between 1993 and 2005, it was obvious that there was a significant drug resistance problem with the circulation of nalidixic acid resistant *S.* Typhi strains. Therefore, we hypothesized there had been an explosion in the incidence of nalidixic acid resistant *S.* Typhi in the years previous to this. We compared nalidixic acid resistant rates in *S.* Typhi strains over a 13 year period from 1993 to 2005.

During the 13 year period, there was a sequential dramatic increase in nalidixic acid resistance (Figure 3. 7). In 1993, 2 out of 61 (3 %) *S.* Typhi isolated from patients in southern Viet Nam was nalidixic acid resistant (MICs to ofloxacin 0.250 µl / mL and 0.125 µl / mL, respectively). Nalidixic acid resistance surged to 88 % (104 / 118) in 1998. It has remained at high levels since, with 91 % (182 / 201) of isolates in 2004. Since 1998, a high proportion of strains showed the combination of MDR and nalidixic acid resistance. The proportional increase in nalidixic acid resistance was highly statistically significant between 1993 and 2005 ($p \leq 0.001$ using Pearson chi-square test with CI 95%).

From 1994 to 2005, the MIC of ofloxacin to southern Vietnamese *S.* Typhi isolates ranged from 0.008 µg / mL to 3 µg / mL (Table 3. 6). The highest MIC_{50} was 1 µg / mL and the highest MIC_{90} was 1.5 µg / mL. The highest MIC value of ofloxacin in 2003 was 1.5 µg / mL, this value increased to 2 µg / mL in 2004 and reached 3 µg / mL in 2005. MIC levels of ciprofloxacin to *S.* Typhi ranged between 0.008 µg / mL and 1µg / mL, and the highest MIC_{50} and MIC_{90} were both 1 µg / mL.

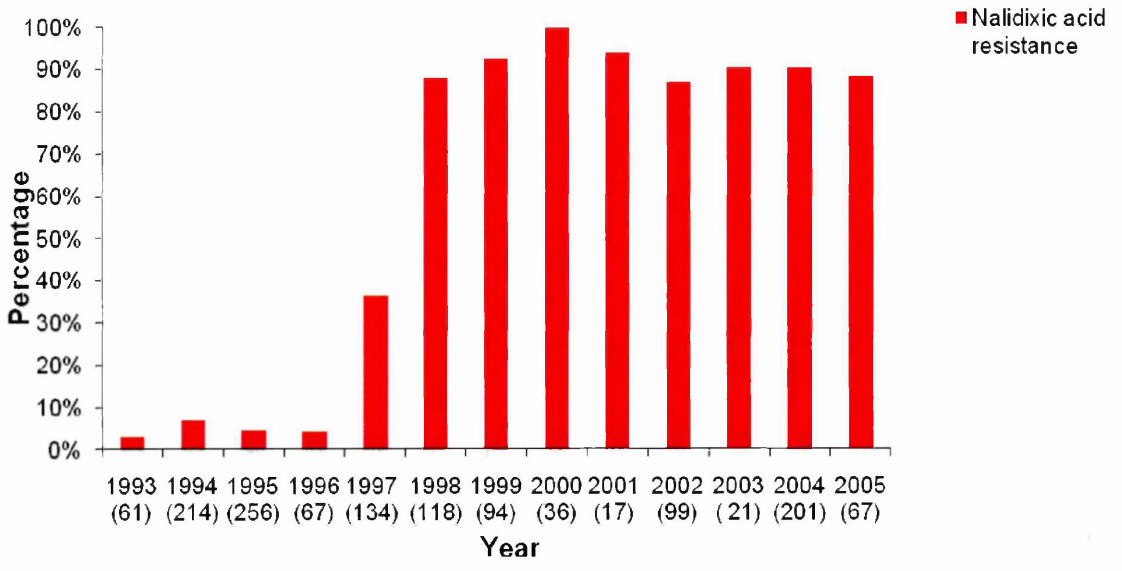


Figure 3.7 Nalidixic acid resistance proportions of *S.* Typhi isolates in southern Viet Nam between 1993 and 2005

Red bars indicate the percentage of nalidixic acid resistant *S.* Typhi isolates which were collected from 18 provinces of southern Viet Nam from 1993 to 2005. the number in brackets is the number of *S.* Typhi isolates in a province, the total number is 1,388 isolates.

A subset of *S.* Typhi isolated from 2003 to 2005 was subjected to MIC testing with gatifloxacin. This was performed as gatifloxacin was being used therapeutically during this period and we wished to know if there was any additional resistance to this antimicrobial [167]. The MIC results ranged from 0.032 µg / mL to 0.5 µl / mL. The MIC_{50} and MIC_{90} were 0.094 µg / mL and 0.19 µg / mL, respectively. Low MIC level to ciprofloxacin and gatifloxacin and intermediate MIC levels of ofloxacin to *S.* Typhi were detected. The trend in southern Viet Nam regarding the population of *S.* Typhi to fluoroquinolones is one of decreasing susceptibility, yet we could identify no strains that exhibited complete resistance to this group of antimicrobials.

Table 3.6 The fluoroquinolone susceptibility of *S.* Typhi isolates in Viet Nam from 1993 to 2005

MIC_{50}, MIC_{90}; Minimum Inhibitory Concentrations at which 50% and 90% of the organisms respectively are inhibited. Range, the minimum and maximum MIC detected in *S.* Typhi isolates. na, not applicable.

Year	No of strain N=569	ofloxacin (µg/mL)			ciprofloxacin (µg/mL)			gatifloxacin (µg/mL)		
		Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
1994	13	0.03-1	0.25	0.25	0.015-0.5	0.06	0.125	na	na	na
1995	20	0.06-2	0.5	1	0.008-0.5	0.125	0.5	na	na	na
1996	38	0.25-1	1	1	0.06-0.5	0.25	0.5	na	na	na
1997	48	0.06-1	1	1	0.015-0.5	0.5	0.5	na	na	na
1998	53	0.008-1	0.5	0.5	0.008-0.5	0.25	0.5	na	na	na
1999	6	0.03-0.5	0.03	0.5	0.008-0.25	0.03	0.25	na	na	na
2000	7	0.03-1	0.03	1	0.008-0.5	0.03	0.25	na	na	na
2001	11	0.06-1	0.5	1	0.015-0.5	0.5	0.25	na	na	na
2002	99	0.5-2	1	1	0.125-1	1	1	na	na	na
2003	21	0.25-1.5	0.75	0.75	0.19-0.5	0.38	0.38	0.094-0.19	0.125	0.19
2004	188	0.023-2	1	1.5	0.125-0.75	0.38	0.5	0.094-0.25	0.125	0.19
2005	65	0.125-3	0.75	1	0.023-0.5	0.38	0.38	0.032-0.5	0.094	0.125

3.3 Discussion

New approaches have been recently developed for rapid screening of *S.* Typhi strains isolated from patients, these include PCR, DNA hybridization probes and Transia cards [28,168]. Although these assays have improved specificity and sensitivity compared to biochemical testing, they also show some disadvantages. The PCR based techniques need to be performed in specialized facilities with skilled technicians and at a higher reagent cost than standard microbiological techniques. In this study culturing on selective media and a short set of chemical tests were first applied for screening *S.* Typhi in a large number of gastrointestinal strains. *S.* Typhi strains were confirmed using serology and API20E tests. Several test steps were required to provide a final identification. This procedure is efficient for examination of *S.* Typhi in term of reliability and cost-efficiency. However, screening large numbers of isolates in this manor is laborious and time consuming and the development of machines for biochemical testing of microorganisms is probable the future of diagnostic microbiology laboratories.

Vi polysaccharide based vaccine has been demonstrated as an effective vaccine to protect children from typhoid fever [169]. However, 7 % (37 / 572) of *S.* Typhi in this study did not express the Vi antigen. Vi negative *S.* Typhi isolates have been described before in Pakistan [166]. Nevertheless, the overall impact of these strains on the bacterial population is unknown. The strains in previously described studies, have been shown to lack the genes which are essential for Vi antigen production [170]. In this study, we did not confirm is the Vi negative strains isolated had the genetic capability to express Vi, or if it was a laboratory artifact. We were able to identify Vi negative *S.* Typhi in three different countries; India, Nepal and Viet Nam. The actual role of Vi antigen is somewhat controversial, evidence suggests that it is important during host

invasion and Vi is considered an important antigen in the pathogenicity of fever *S.* Typhi [171]. Looney showed the Vi inhibits complement mediated lysis and phagocytosis [172]. Hirose additionally demonstrated the presence of Vi on the surface of *S.* Typhi reduces TNFα secretion, which is a marker of macrophage activation [173]. However, Vi negative *S.* Typhi strains can cause infection, outbreaks caused by Vi negative *S.* Typhi have been described in India [174]. Furthermore, *S.* Paratyphi A, which causes an indistinguishable disease to *S.* Typhi, does not express the Vi antigen. Potentially, if *S.* Typhi has the ability to switch phase Vi antigen expression during systemic infection, it may allow the organism to evade the host immune response. The overall effect of Vi remains contentious, because Vi negativity can arise during *in vitro* passage, further investigation is required to understand the mechanism of this phenomenon; the deployment of Vi vaccinations in areas where Vi negative strains may circulate may be detrimental to the overall protective efficacy of the vaccine, yet the overall impact of Vi negative strains is not widely understood.

The majority of *S.* Typhi strains isolated globally have fluoroquinolone MICs which are usually within the susceptible range of the interpretive criteria of the CLSI. However, a growing body of clinical and microbiological evidence indicates that nalidixic acid resistant *S.* Typhi also exhibit a decreased clinical response to treatment with fluoroquinolones [175]. Whilst, our data does not include patient treatment and therapy responses, we hypothesize that the increase in nalidixic acid resistance strains will have a large impact on fluoroquinolone therapy in places where these strains predominate.

According to WHO (2005), among 1,055,178 diarrheal cases reported in Viet Nam in 2002, 6,936 cases were typhoid fever [40], whilst a report in 1995 suggested that the incidence of typhoid cases was about five times higher (30,901 cases) [176].

Approximately 90 % of strains that were compared here were isolated in southern Viet Nam which consists of 17 provinces and includes 39 % of the total population of Viet Nam [42]. In 1993, during an initial outbreak of MDR *S.* Typhi in Kien Giang province in the south of Viet Nam, the fluoroquinolone antimicrobials were introduced for the treatment of typhoid fever [164]. Since 1993, the proportion of MDR *S.* Typhi in the south of Viet Nam has remained at high levels and there has been a dramatic increase in nalidixic acid resistance. Although there has been a reduction in the number of typhoid cases seen in southern Viet Nam, the severity of disease has increased (measured by prolonged fever and antimicrobial therapy). In our clinical studies typhoid was commonly caused by MDR and nalidixic acid resistant *S.* Typhi (63 – 78 %) [127,162,163]. The severity of infection was directly related to the resistance profile of the infection organism such as high failure cure rate, prolonged for fever clearance time and high feacal carriage rate [114, 115, 172, 173].

In 1998, five years after ofloxacin and ciprofloxacin become widely available in an uncontrolled market, 88 % of *S.* Typhi isolates were resistant to nalidixic acid, and this proportion increased to 90 % by 2004. The combination of MDR and nalidixic acid resistance is particularly problematic in Viet Nam, because it severely restricts the therapeutic options for patients with typhoid fever. Although MDR - nalidixic acid resistant strains were a serious problem in the south, no such strains were detected in the North of Viet Nam [177]; and there have been no reports of MDR - nalidixic acid resistant strains in central Viet Nam. The spread of MDR - nalidixic acid resistant strains in the south of Viet Nam may be related to an individual clone undergoing positive selection and consistent transmission. The overall incidence of typhoid is higher in southern Viet Nam (with respect to the other areas of Viet Nam), thus high transmission routes may permit clonal selection. This is potentially due to the overall

water density in the Mekong Delta region that may aid transmission. An additional possibility for the emergence of MDR - nalidixic acid resistant strains in the south of Viet Nam may be the uncontrolled nature of antimicrobial distribution, allowing self-medication at a level which diminishes symptoms but does not prevent shedding and onward transmission.

Patients infected with nalidixic acid resistant *S.* Typhi show poor clinical responses, including, high failure rates (up to 36 %), prolonged for fever clearance and protracted faecal carriage when treated with both old (e.g ofloxacin) and new (e.g. ciprofloxacin) generation fluoroquinolones [120,121,178,179]. Fortunately, neither azithromycin nor ceftriaxone resistance was identified in Viet Nam, therefore, they would seem to be the treatment of choice for typhoid patients since the majority of strains showed nalidixic acid resistance and reduced susceptibility to fluoroquinolone. However, the cost of treatment for drug resistant strain with ceftriaxone is twice as costly in comparison with treatment for a patient infected with a sensitive strain [180]. Owing to the dramatic nature of antimicrobial resistance in most bacterial pathogens there is an obvious requirement for new, cost effective antimicrobial agents to be developed.

The antimicrobial resistance data from southern Viet Nam is complemented by the results of the cross-sectional data from 7 Asian countries; Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan. From our collection, no antimicrobial resistant *S.* Typhi was detected in China or Indonesia, apart from 14 % (3 / 21) of strains in China being resistant to azithromycin. Wang *et al.* (2005) [34] reported similar antimicrobial resistance profiles, by studying 40 *S.* Typhi strains from China challenged with 12 antimicrobial agents. Azithromycin resistance in *S.* Typhi in China and India (7 %) raises concern, as it is the antimicrobial agent currently is the drug of choice for

nalidixic acid resistant *S.* Typhi strains [165,167]. Clinically, patients may respond satisfactorily to azithromycin even if isolates have intermediate resistance, according to current guidelines [19].

Antimicrobial resistance in Indonesia was not seen to be a problem (when compared to other areas). This is consistent with the finding of Hatta *et al.* [181], even though the sample size was small (17 strains), these finding are the first described with respect to the phenomenon. However, Hatta *et al.* has described more recent trends of antimicrobial resistance in Indonesia which appears to be similar to other locations in Asia [181].

Reduced susceptibility to ceftriaxone was found only in Nepal, the MIC ranged from 0.002 μg / mL to 6 μg / mL and MIC_{50} and MIC_{90} were 0.094 μg / mL and 3 μg / mL respectively and no defined ceftriaxone resistance was detected among *S.* Typhi isolates. Some finding in Kuwait have shown that *S.* Typhi producing extended spectrum beta lactamases (ESBL) are circulating [182]. We did not confirm if the strains isolated from Nepal were ESBL producing, due to them only have reduced susceptibility. ESBL producing *S.* Typhi are rare and there are only limited reports [183,184],[185].

While in southern Viet Nam the MDR phenotype of *S.* Typhi has remained at high levels over the last 13 years, there have been reports of a return to chloramphenicol sensitive *S.* Typhi isolates in some regions [186,187]. In our study the prevalence of chloramphenicol resistance remained high in almost all Asian countries (with the exception of China and Indonesia) (18 % in Laos, 19 % in Nepal, 26 % in India 2003, which reduced to 14 % in 2006, 26.5 % in Pakistan, and 40 % in Bangladesh).

Ampicillin resistance remained at a low level in Laos (20 %), Pakistan (17 %), and reduced from 26 % to 11 % in India in 2003 and 2006, respectively. Ampicillin resistance was particularly high in southern Viet Nam (49 %) and Bangladesh (100 %). The correlation between sulfamethoxazole - trimethoprim (co-trimoxazole) resistance and chloramphenicol resistance needs to be further defined and may be co-transferred on the same plasmid backbone.

The percentage of MDR *S.* Typhi decreased in India between 2003 and 2006. This finding was consistent with a previous report [188]. A study in New Delhi (2005) reported 60 % of MDR typhoid cases detected in a cross sectional study of samples from 5 different institutes. The increase in chloramphenicol sensitive isolates is assumed to be in response to the shift in using chloramphenicol, ampicillin and co-trimoxazole to fluoroquinolones as a first choice of typhoid therapy [127,189].

In 2002 to 2004, all countries in the region, with the exception of China and Laos, faced a problem of nalidixic acid resistance, with southern Viet Nam as a particular hot spot. Our findings were supported by a report of Capoor, 51 % of *S.* Typhi in the study were resistant to nalidixic acid, and nearly 100 % of strains had reduced susceptibility to ciprofloxacin [190]. The observation of *S.* Typhi ciprofloxacin resistance (2 / 259, 1 %) in India and (6 / 149, 4 %) in Nepal in this study, together with observations of high-level ciprofloxacin resistance in India, Tajikistan and Bangladesh [191-194] might be the prelude to a worsening drug resistance problem in Asia. The increase in gatifloxacin MIC values raised more concerns on the therapy alternatives in typhoid treatment. However, a correlation in increasing MIC between fluoroquinolones has not been clarified, and unrelated nalidixic acid sensitivity and decreased ciprofloxacin susceptibility has been reported in Europe [195].

Whilst the collection amassed was the most comprehensive available, there are still limitations which may bias the interpretation of the data. The sampling is specifically biased to the location of the centers involved in the study. It is clear that *S.* Typhi population is dynamic and may fluctuate within countries and within local regions. Our finding may be biased by analyzing a limited sample size in each of the communities selected. There may also be different drug usage in the different locations; this in turn may affect the drug susceptibility pattern. Nonetheless, this work respects an exhaustive snapshot of the changing resistance patterns of *S.* Typhi in Asia.

In conclusion, the emergence and persistence of MDR and nalidixic acid resistant *S.* Typhi is a major problem across Asia. We suggest that outbreaks of fluoroquinolone resistant *S.* Typhi may be common in future years, owing to the pressure placed on fluoroquinolonses. Although the reduced susceptibility to ceftriaxone and resistance to azithromycin were found, it was rare. Thus, these antimicrobial agents would be safe to use clinically in order to potentially eliminate the development of fluoroquinolone resistance. No drug has ever been developed specifically for typhoid fever and there are very few potential targets in the *Salmonellae* against which new drugs could be designed [196]. Treating clinicians should consider the ongoing antimicrobial resistance problem and use current drugs more efficiently and use the best drugs available in the most sensible regime in order to prevent further resistance. Policies for the drug markets, especially in Viet Nam, where drugs are sold casually, should be emphasized in order to eliminate the development of drug resistance and the using of antimicrobial in farming should be legislated. Enhancing both education of health professionals and the community to ensure appropriate antimicrobial usage and to avoid self-medication respectively would be other urgent strategies [177]. The knowledge of the extent of

antimicrobial resistance should be an important issue when discussing the implementation of a comprehensive typhoid vaccination strategy.

4. Molecular mechanisms of quinolone resistance and reduced susceptibility to fluoroquinolones in *S.* Typhi

4.1 Introduction

Nalidixic acid resistance and reduced susceptibility to fluoroquinolones in *S.* Typhi correlates with treatment failure and has been reported in Viet Nam and internationally [165,192,197-201]. However, fluoroquinolones resistance in *S.* Typhi is relatively uncommon compared to the resistance frequency in other *Enterobacteriaceae* [99]. As a result, newer generations of fluoroquinolones remain the drug of choice for typhoid treatment [167,202]. It has been suggested that a number of different fluoroquinolones resistance mechanisms may play a role with this phenotype in the *Salmonellae* species [203]. As multiple mechanisms may be involved in fluoroquinolone resistance, an extensive study in the mechanisms of nalidixic acid resistance and reduced susceptibility to fluoroquinolones in *S.* Typhi is necessary.

All fluoroquinolones have an identical mechanism of action [204] implying that resistance to one fluoroquinolone will confer resistance to other fluoroquinolones [203]. They act by inhibiting the topoisomerase genes leading to inhibition of DNA replication. Four main mechanisms conferring fluoroquinolone resistance in *Salmonellae* have been extensively studied, wherein mutations in the genes encoding DNA gyrase and topoismerase IV are the most commonly observed. Decreased accumulation of drug molecules due to decreased fluoroquinolone uptake [205,206], increased active efflux pump activity [108,207] and more recently described plasmid mediated quinolone resistance (PMQR) are the other mechanisms accounting for fluoroquinolone resistance in *Salmonellae*. While, plasmid-mediated quinolone resistance of the Qnr has been widely observed in other *Enterobacteriaceae* worldwide, there are limited reports in *Salmonellae* [208-211].

Nalidixic acid resistance and reduced susceptibility to fluoroquinolones in *S.* Typhi strains has linked to point mutations within codon 83 and codon 87 in the QRDR (Quinolone Resistance Determining Region) of *gyrA* gene. Some mutations have also been observed outside the QRDR of *gyrA* gene [190,212,213] and a high level of fluoroquinolone resistance could possibly be a combination of mutations in the target regions of the *gyrA* and *parC* genes [214].

Several methods have been reported for the detection of point mutations in bacterial genes including those within the QRDR of the *gyrA, gyrB, parC* and *parE* (Table 4.1). In this study we firstly applied the conventional dideoxy DNA sequencing to detect alterations in the target enzymes of *S.* Typhi isolates. More recently, due to improvements in DNA sequencing technology we have employed pyrosequencing for rapid screening of a large amount of *S.* Typhi strains for known mutations in *gyrA* gene. Here we have screened 462 strains (337 from southern Viet Nam, 121 from India and 4 from Pakistan) and 14 nalidixic acid susceptible *S.* Typhi (2 from China, 5 from India, 2 from Indonesia, 2 from Pakistan and 3 from Viet Nam) to identify the mechanisms of quinolone resistance and reduced susceptibility to fluoroquinolones in *S.* Typhi.

Table 4.1 Methods applied to investigate mutations in topoisomerase genes

Method	Advantage	Disadvantage	Reference
Single-strand conformational polymorphism (SSCP)	Novel mutation detection	Technical complication	[215]
Rapid mismatch amplification mutation assay (MAMA)	Simple and rapid alternative to SSCP and DNA sequencing	Using mutation-specific primers, not detect other sequence variations	[216]
PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)	Detect known mutation		[217]
LighCycler based PCR-hybridization *gyrA* mutation assay [218]	A simple and rapid, several mutations can be detected, quick screening method	Probes have proved difficult to use	[219]
DNA microarray	Novel mutation detection with complete confidence		[211]
Allele-specific PCR and RFLP (AS-PCR-RFLP)	Rapid, high- throughput, detecting novel mutation		[220,221]
DNA sequencing	Gold standard method, detect known and unknown mutations	Available only in laboratories that have DNA sequencing capability	[222]
Denaturing High-performance Liquid Chromatography (DHPLC)	Detect known and novel mutations	Intensive lab capacity	[223]
Pyrosequencing	Easy to perform, less expensive, less labor intensive and time-consuming. Rapid analysis large number of sample		[224]

4.2 Results

4.2.1 DNA sequence analysis of QRDR of gyrase and DNA topoisomerase IV encoded genes and effects of the mutations on fluoroquinolone susceptibility

Four hundred and seventy six *S.* Typhi strains were subjected to molecular analysis. DNA was extracted using two methods, CTAB (Cetyl Trimethyl Ammonium Bromide) method and Promega kit as described in section 2.7.1. In comparison, CTAB method produced more DNA than the Promega method. However, the Promega kit produced better DNA quality and was easier to perform, resulting from the use of the ready-to-use reagents. DNA extraction using Promega kit then was reproducible and not time consuming. Thus, depending on reagents availabilities, Promega kit was considered preferable.

DNA was quantified before using as a template for PCR reactions. A 347 bp fragment of *gyrA* (Figure 4.1), a 345 bp fragment of *gyrB*, a 270 bp fragment *parC* and a 240 bp fragment *parE* of the QRDR (Quinolone Resistant Determining Region) were amplified by PCR.

The PCR amplicons from the gyrase and TopoIV genes including *gyrA* (100 strains), *gyrB* (85 strains), *parC* (146 strains) and *parE* (62 strains) were directly sequenced by conventional sequencing in the first part of the experiment. The remainder of samples (376 strains) was screened for *gyrA* mutations using pyrosequencing since the available of new robust sequencing facility in the Unit. The Sanger sequencing using the CEQ 8000 allows nucleotide sequencing length of up to approximately 700 bp. In comparison with other methodologies, this is an ideal technique to detect novel mutations in such length of fragment (Table 4.1). However, sequencing using the CEQ 8000 system can only be performed after one PCR reaction, one step of PCR amplicon

purification, one sequencing reaction followed by a capillary gel electroforesis. PCR amplicons have to be purified to avoid any possible inhibition during the sequencing process. The mutations in *gyrA* and *parC* genes detected using CEQ8000 system are represented in Figure 4. 2.

Figure 4.1 A representative agarose gel of the PCR amplicons for the QRDR fragment of the *gyrA* gene (347bp)

Lanes 1 to 12 are *gyrA* gene PCR amplicons of *S.* Typhi DNA isolated from An Giang province, southern Viet Nam. The DNA from *S.* Typhi isolates that were amplified are lane 1; AG 146, lane 2; AG 152, lane 3; AG 160, lane 4; AG164, lane 5; AG 165, lane 6; AG 166, , lane 7; AG 168, lane 8; AG 169, lane 9; AG 170, lane 10; AG 176, lane 11; AG 177, lane 12; AG 178. Neg; negative control performed using the same PCR mix with distilled water instead of DNA sample.

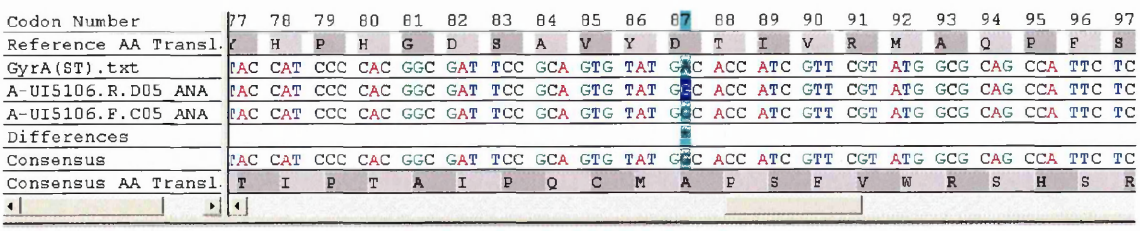


Figure 4.2 A representative DNA sequence alignent from the *gyrA* gene of a mutant *S.* Typhi strain

The alignments were performed using CEQ 2000 XL software with the reference sequence was CT 18 (Gene ID 1248826) from NCBI webpage (www.ncbi.nlm.nih.gov). The strain amplified and sequenced here is strain UI 5106 from Laos and contains the substitution Asp87→Gly (replacing GAC with GGC).

Pyrosequencing latterly appeared to be a robust assay for short sequence analysis. Also the assay was a reliable solution for rapid screening of known mutations. The

sequencing reaction was performed directly after amplification of the target fragment. The limitations of pyrosequencing are that the technique is only applicable for fragments with less than 70 bp and the software has limitations in analyzing multiple mutations between 15 nucleotide sequences. However, the results can be analyzed by comparing the conventional trace files or using appropriate software, such as the alignX tool of the Vector NTI Suite 7 programme.

The percentage of each mutation observed in the *gyrA* and *parC* genes is depicted in Figure 4.3. Seven different types of mutation were detected in the 476 strains analyzed. The most prevalent amino acid substitution was Ser 83 → Phe (TCC → TTC) observed in 407 / 462 (88 %) strains. Forty seven isolates (10 %) had a substitution at codon 83 changing Ser → Tyr (TCC → TAC). Two isolates showed an Asp 87 → Gly (GAC → GGC) substitution and two isolates had an Asp 87 → Ala (GAC → GCC) substitution. Two Vietnamese *S.* Typhi strains had a double amino acid substitution in the *gyrA* gene; these were isolates DT 18 (Ser 83 → Phe and Asp 87 → Gly) and AG 152 (Ser 83 → Phe and Asp 87 → Asn). Two isolates from India, C 2114 and C 2115 had triple amino acid substitutions including double mutations in *gyrA* (Ser 83 → Phe and Asp 87 → Gly) and a single mutation in *parC* (Ser 80 → Ile). All amino acid substitutions that were found are presented in Table 4. 2.

S. Typhi strains observed for *gyrA* mutations were also analyzed for mutations in the QRDRs of *gyrB* (85 strains), *parC* (146 strains) and *parE* (62 strains) genes [147,225]. No mutations were detected in the *gyrB*, *parC* or *parE* genes of the strains analyzed apart from 2 strains which had a substituted Ser 80 → Ile in the *parC* gene. One hundred and fifty two isolates were additionally screened for the presence of plasmid-

mediated quinolone resistance genes *qnrA* and *qnrS* following the previous described method [210]; none were detected in these *S.* Typhi screened.

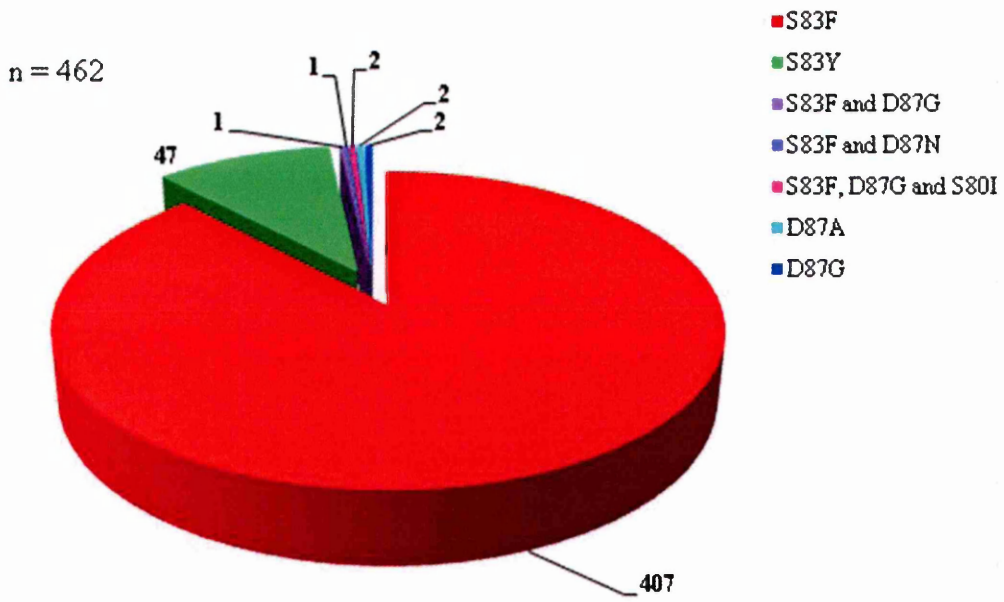


Figure 4.3 The proportions of amino acid substitutions detected in the *gyrA* and *parC* genes of *S.* Typhi strains from Asia

Colours depict the proportion of each mutation type found in the collection, red; the number of *S.* Typhi strains with the substitution Ser83 → Phe (S83F), green; the number of *S.* Typhi strains with the substitution Ser83 → Tyr (S83Y), light purple; the number of *S.* Typhi strains with the substitution Ser83 → Phe and D87 → Gly (S83F and D87G), purple; the number of *S.* Typhi strains with the substitution Ser83 → Phe and Asp87 → Asn (S83F and D87N), pink; the number of *S.* Typhi strains with the substitution Ser83→Phe, Asp87→Gly (D87G) and Ser80→Ile (S80I), light blue; the number of *S.* Typhi strains with the substitution Asp87→Ala (D87A), blue; the number of *S.* Typhi strains with the substitution Asp87→Gly (D87G).The number depicts the absolute number of each mutant which were detected in 462 *S.* Typhi strains of the Asian collection.

Table 4.2 The amino acid substitutions detected in *gyrA* and *parC* of *S.* Typhi isolates from Asia

Pink letters represent the amino acids found in the *gyrA* and *parC* genes (STY 2499) in *S.* Typhi CT 18, which was used as a reference sequence [66]. Red letters represent the amino acid substitutions in the *gyrA* and *parC* genes of the *S.* Typhi strains which were isolated in Asian countries. The nucleotide changes corresponded to the amino acid changes in *gyrA* gene are S83F (TCC→TTC); S83Y (TCC→TAC); D87G (GAC→GGC); D87A (GAC→GCC); D87N (GAC→AAC) and the amino acid change in *parC* gene is S80I (TCG→TAC). The numbers represent the amino acid loci in the genes.

Type	Number of strain (n=476)	Amino acid sequence	
		gyrA	*parC*
		70 83 87	70 80
S83F	407	VGDVIGKYHPHGD**F**AVYDTIVRMAQP	
S83Y	47	VGDVIGKYHPHGD**Y**AVYDTIVRMAQP	
Wild	14	VGDVIGKYHPHGDSAVYDTIVRMAQP	VLGKYHPHGDSACYEAMVLMAQPFSY
D87G	2	VGDVIGKYHPHGDSAVY**G**TIVRMAQP	
D87A	2	VGDVIGKYHPHGDSAVY**A**TIVRMAQP	
S83F and D87G	1	VGDVIGKYHPHGD**F**AVY**G**TIVRMAQP	
S83F and D87N	1	VGDVIGKYHPHGD**F**AVY**N**TIVRMAQP	
S83F and D87G and S80I	2	VGDVIGKYHPHGD**F**AVY**G**TIVRMAQP	VLGKYHPHGD**I**ACYEAMVLMAQPFSY

4.2.2 MIC comparison of quinolones and fluoroquinolone susceptibilities of *S.* Typhi strains with and without mutations in the *gyrA* and *parC* genes

The MICs to nalidixic acid and ofloxacin of strains carrying the different mutations were compared. Overall, the MIC values to nalidixic acid were significantly increased when groups with mutations were compared to non - mutants ($p < 0.001$). The MIC range for nalidixic acid varied from 2 µg / mL to 256 µg / mL for the mutants and from 0.012 µg / mL to 256 µg / mL for the non - mutants as shown in Figure 4. 4.

The MIC to ofloxacin in the nalidixic acid resistant *S.* Typhi mutants varied amongst the assorted mutant groups and ranged from 0.023 µg / mL to 12 µg / mL across all mutants (Figure 4.5). The MIC to ofloxacin of strains with single mutations varied within the susceptible range, from 0.023 µg / mL to 2 µg / mL (according to the CLSI guidelines, < 2 µg / mL for sensitive strains and > 8 µg / mL for resistant strains). The MIC to ofloxacin was significantly increased in mutants when compared to the non - mutants (p value < 0.001). However, this was not observed between non - mutants and the single mutation groups or amongst the differing single mutants themselves. This discrepancy is dependant on limited numbers of isolates in the double and triple mutant groups. Generally, double mutations caused a decreased susceptibility to ofloxacin in comparison with single substitutions (Figure 4.6).

The MIC values of the two double mutants (DT 18 and AG 152) to ofloxacin were 2 µg / mL and 3 µg / mL respectively. A comparison between the two double mutations found that strain AG152 with S83F and D87N mutations demonstrated increased resistance to ofloxacin with respect to strain DT 18 with S83F and D87G substitutions. Strains C 2114 and C 2115 with triple mutations, a combination of double *gyrA* mutations (S83F and D87G) and single *parC* (S80I) mutation caused the highest level

of resistance to ofloxacin (Figure 4. 6) with ofloxacin MICs of 6 µg / mL and 12 µg / mL, respectively.

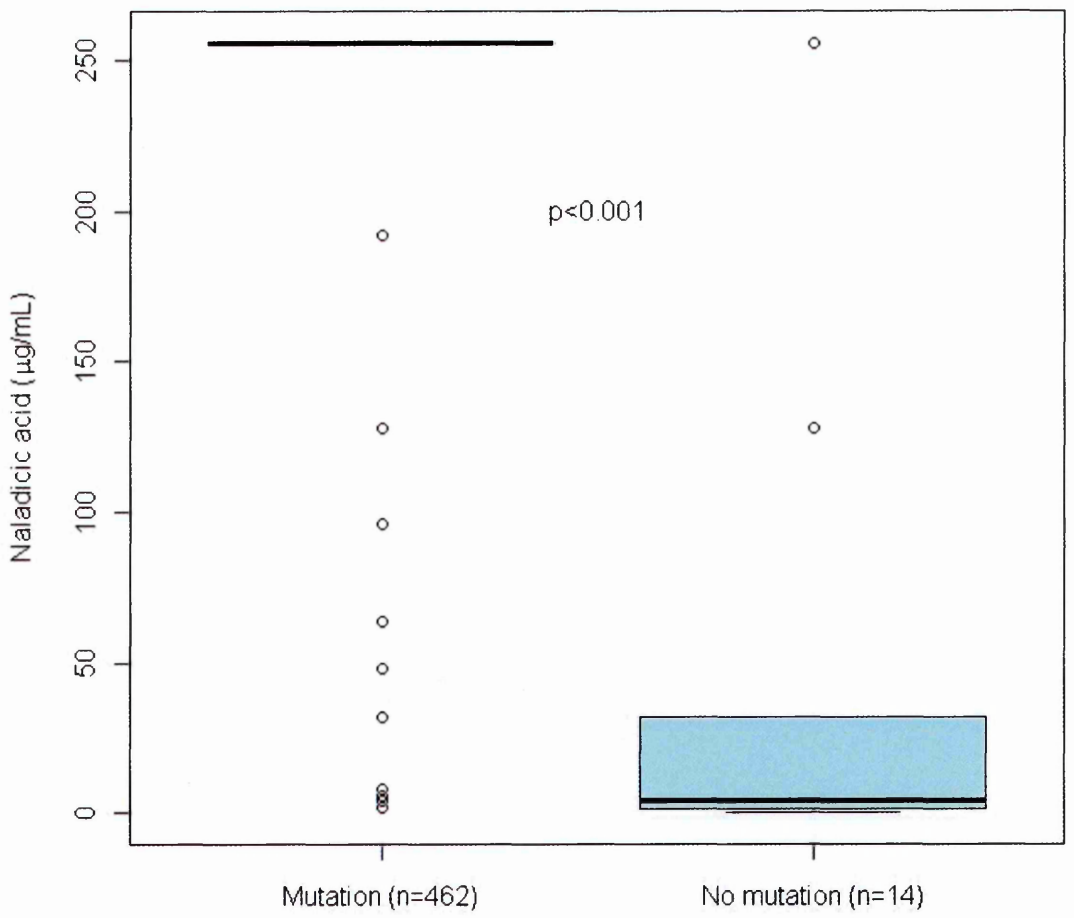


Figure 4.4 The correlation between MIC to nalidicic acid between the no mutation and mutation groups in the *gyrA* gene of *S.* Typhi strains

The median value (with the interquartile range represented by the box at the 5^{th} – 95^{th} percentile by error bars) of the MIC of nalidixic acid of two *S.* Typhi groups, no mutation and mutation. Dots represent the outliers. n; the number of the *S.* Typhi was observed. The difference of MIC value between no mutation and mutation *S.* Typhi groups were evaluated by Wilcoxon test. (CLSI guideline MIC < 16 µg / mL for sensitive and MIC > 32 µg / mL for resistant strains).

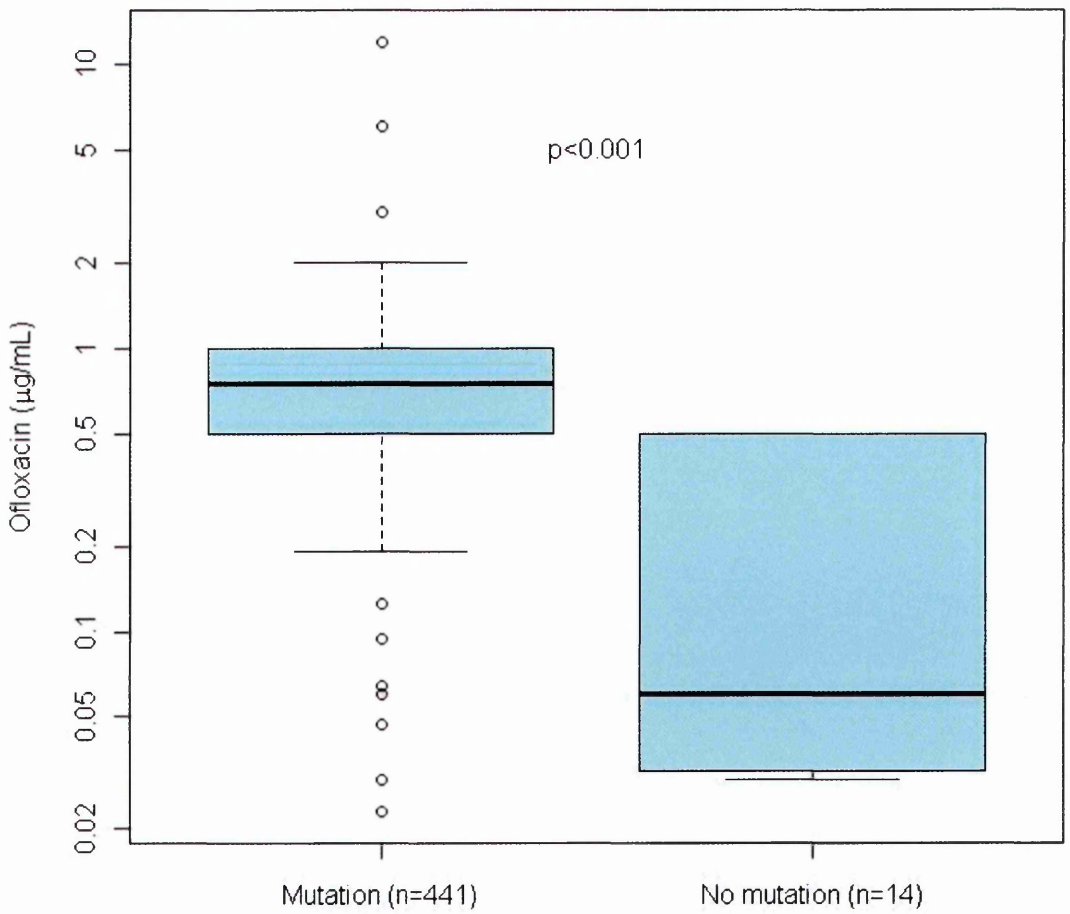


Figure 4.5 The MIC comparison to ofloxacin between the no mutation and the mutation groups of *S.* Typhi isolates in Asian countries

The median value (with the interquartile range represented by the box at the 5^{th} – 95^{th} percentile by error bars) of the MIC of ofloxacin of two *S.* Typhi groups, no mutation and mutation. Dots represent the outliers. n; the number of the *S.* Typhi strains observed. The difference in MIC value between no mutation and the mutation *S.* Typhi groups was evaluated by Wilcoxon test. The y axis represents the $\log_{10}$ value of ofloxacin. (According to CLSI guidelines, the MIC breakpoints are < 2 μg / mL for OFX (sensitive) and > 8 μg / mL for OFX (resistant)).

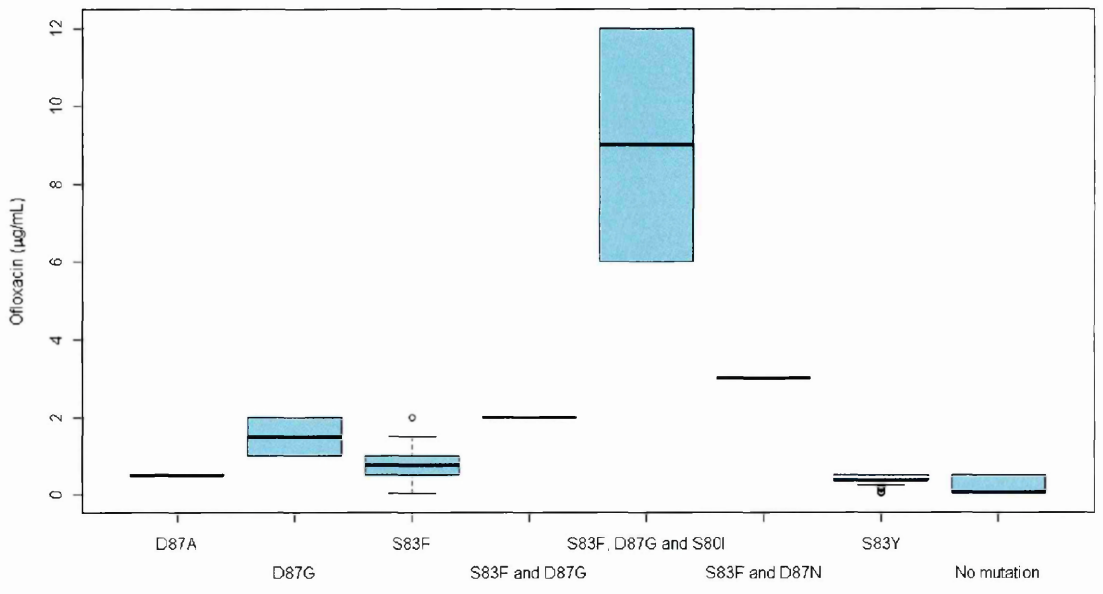


Figure 4.6 The effect of multiple mutations in the *gyrA* and *parC* genes on MIC to ofloxacin in *S.* Typhi isolates from Asian countries

The median value (with the interquartile range represented by the box at the 5^{th} – 95^{th} percentile by error bars) of the MIC to ofloxacin of different mutations and no mutation groups. Dots represent the outliers. The y axis represents the $\log_{10}$ value of the MICs ofloxacin.

The MICs to ciprofloxacin were also compared between the mutant and non - mutant groups. The mutants demonstrated a significant increase in MIC to ciprofloxacin with $p < 0.001$ using the Wilcoxon test (Figure 4.7). Mutations in *gyrA* gene again correlated with a reduced susceptibility to ciprofloxacin. Amongst the differing single mutation strains, there was no significant difference in the MICs to ciprofloxacin (MIC ranging from 0.006 μg / mL to 0.75 μg / mL). However, the MICs of the single substitution D87A (mean, 0.093 μg / mL) were slightly lower than that of the other single mutation groups. The MIC values of the substitutions at position 83 demonstrated the most variation when compared to strains with other substitutions. The ciprofloxacin MICs ranged from 0.06 μg / mL to 0.75 μg / mL for the strains with the S83F substitution and from 0.006 μg / mL to 0.38 μg / mL for those strains with the S83Y substitution. The difference in MIC levels to ciprofloxacin of this group may not only be the result of a single substitution but also other secondary contributing factors that were not detectable in these assays. There was no significant difference in MICs to ciprofloxacin within the double mutants, and similar to the MICs to ofloxacin, the triple mutant had the highest level of resistance to ciprofloxacin with MICs of 4 μg / mL for the strain C 2114 and 6 μg / mL for the strain C 2115 (Figure 4.8).

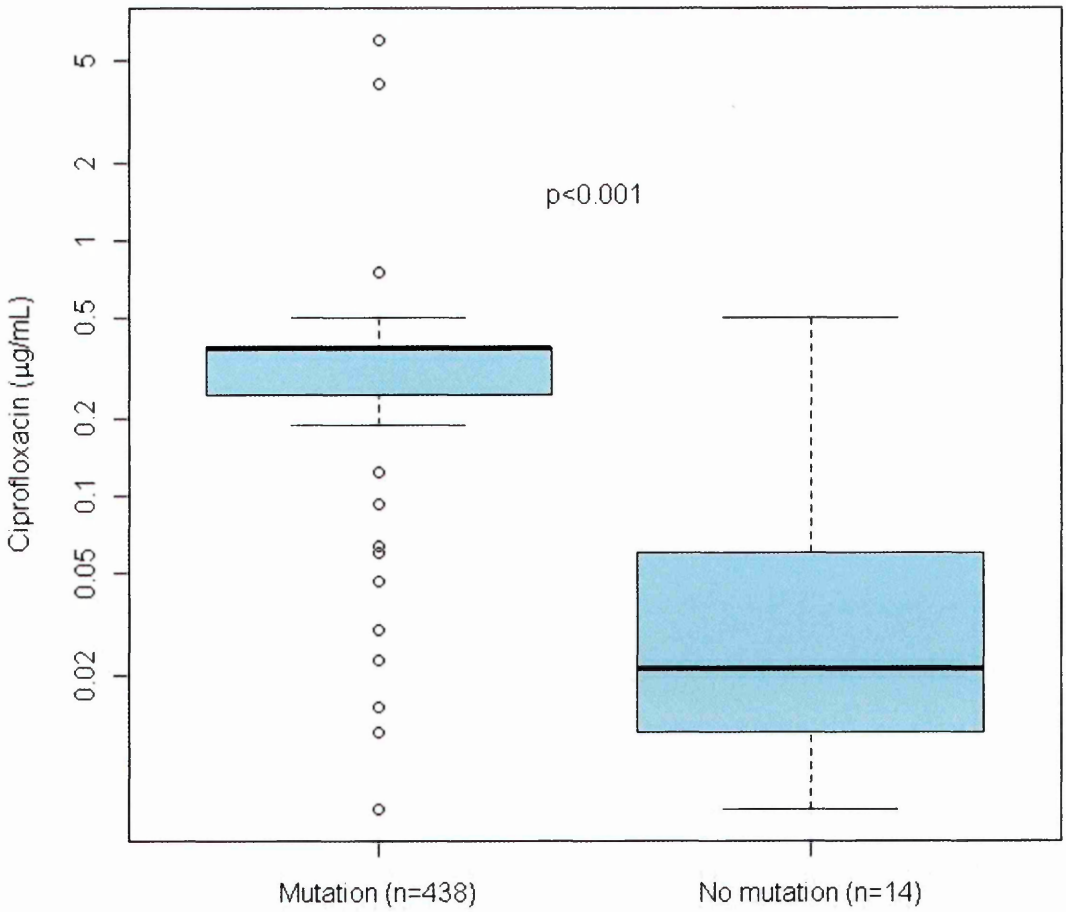


Figure 4.7 The MIC comparison to ciprofloxacin between the no mutation and the mutation groups of *S.* Typhi isolates in Asian countries

The median value (with the interquartile range represented by the box at the 5^{th} – 95^{th} percentile by error bars) of the MIC to ciprofloxacin of two *S.* Typhi groups, no mutation and mutation. Dots represent the outliers. n; the number of the *S.* Typhi strains tested. The difference of MIC value between non - mutant and mutant *S.* Typhi groups was evaluated by Wilcoxon test. The y axis represents the $\log_{10}$ value of the MICs ciprofloxacin. According to CLSI guideline, the MIC breakpoints are < 1 µg / mL for ciprofloxacin sensitive and > 4 µg / mL for ciprofloxacin resistant strains).

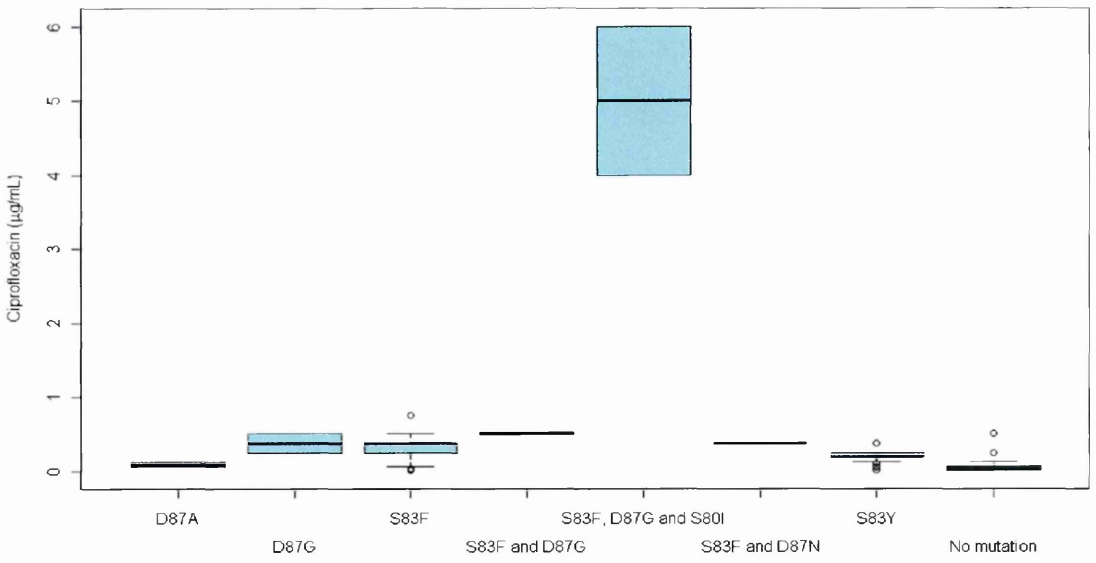


Figure 4.8 The effect of multiple mutation in the *gyrA* and *parC* genes on MIC to ciprofloxacin in *S.* Typhi isolates from Asian countries

The median value (with the interquartile range represented by the box at the 5^{th} – 95^{th} percentile by error bars) of the MIC to ciprofloxacin of different mutation *S.* Typhi groups. Dots represent the outliers. The y axis represents the $\log_{10}$ of the MIC value of ciprofloxacin.

MIC levels were also assessed in all mutant groups to the newest therapeutic fluoroquinlone, gatifloxacin. According to the CLSI guidelines [141], all *S.* Typhi isolates were susceptible to gatifloxacin, the MIC to gatifloxacin ranged from 0.032 µg / mL to 0.5 µg / mL in all tested strains. There was a significant difference in MIC to gatifloxacin between the mutant strains and the non - mutant strains, $p < 0.001$ using Wilcoxon test (Figure 4.9). There was no significant difference in MIC level to gatifloxacin amongst the differing mutant groups with the exception of the triple mutants (MIC 2 µg / mL for the strain C 2114 and MIC 2 µg / mL for the strain C 2115) (Figure 4.16).

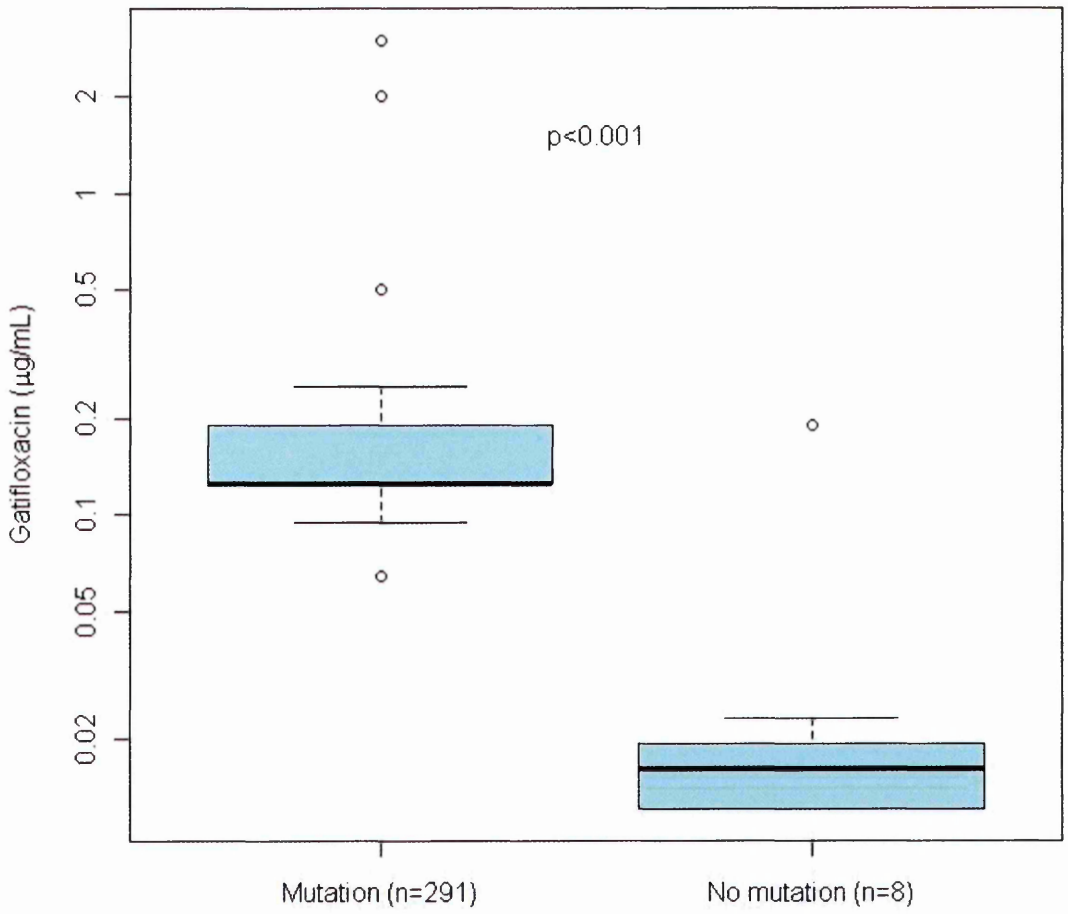


Figure 4.9 The MIC comparison to gatifloxacin between the mutation and the no mutation groups of *S.* Typhi isolates in Asian countries

The median value (with the interquartile range represented by the box at the 5^{th} – 95^{th} percentile by error bars) of the MIC of gatifloxacin of two *S.* Typhi groups, no mutation and mutation. Dots represent the outliers. n; the number of the *S.* Typhi strains tested. The difference of MIC value between the non - mutant and the mutant *S.* Typhi groups was evaluated by Wilcoxon test. The y axis represents the $\log_{10}$ value of the MICs gatifloxacin. According to CLSI guideline, the MIC breakpoints are < 2 µg / mL for gatifloxacin sensitive and > 8 µg / mL for gatifloxacin resistant strains).

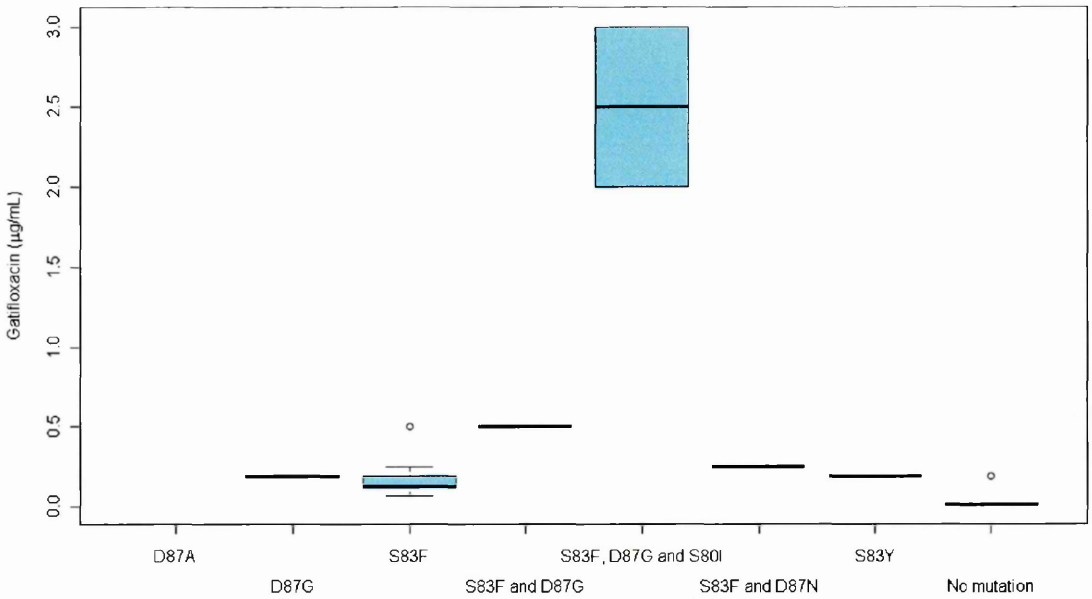


Figure 4.10 The effect of multiple mutation in the *gyrA* and *parC* genes on MIC to gatifloxacin in *S.* Typhi isolates from Asian countries

The median value (with the interquartile range represented by the box at the 5^{th} – 95^{th} percentile by error bars) of the MIC of gatifloxacin of different mutation *S.* Typhi groups. Dots represent the outliers. The y axis represents the $\log_{10}$ of the MIC value of gatifloxacin.

4.2.3 The correlation of increasing MIC of differing fluoroquinolones in *S.* Typhi mutants and non - mutants

The MIC data of all strains for ciprofloxacin, ofloxacin and gatifloxacin were compared to attempt to identify any linear correlation between MIC patterns of the three tested antimicrobials as shown in Figure 4.11, 4.12 and 4.13. There was a statistically significant linear correlation between all three antimicrobials tested using Pearson test for linear correlation by R software version 2.0, whereby an increase in MIC to either gatifloxacin, ciprofloxacin or ofloxacin correlated with a direct increase to a corresponding fluoroquinolones. A direct relationship was observed most clearly between ciprofloxacin and ofloxacin, where gradual increases in MICs were reflected the rise in the other two antimicrobials (Figure 4.12). The linear relationship in MIC increase between the different fluoroquinolones was also observed between ciprofloxacin and gatifloxacin and between ofloxacin and gatifloxacin; however, this effect was less pronounced (Figure 4.11 and Figure 4.13). An increase in MICs with gatifloxacin generated a step wise increase in MIC with both ciprofloxacin and ofloxacin. This effect may be due to the differing activities and binding location of the gatifloxacin when compared to both ofloxacin and ciprofloxacin.

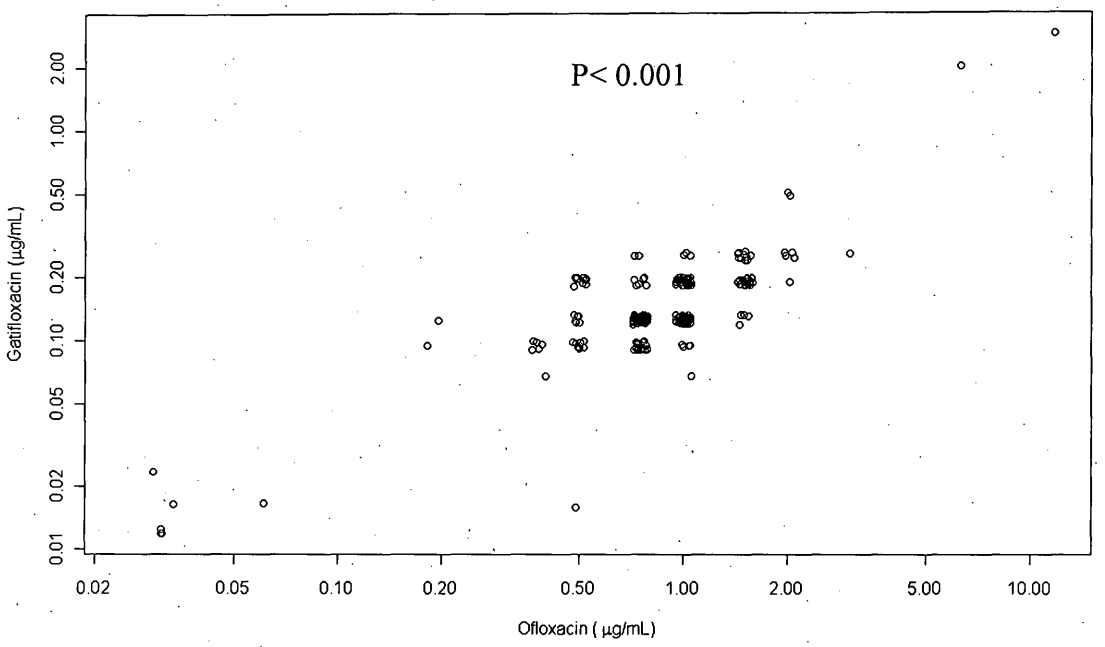


Figure 4.11 The relationship of ofloxacin and gatifloxacin MICs of *S.* Typhi mutants and non - mutants from Asian countries

The y axis represents the $\log_{10}$ MIC value of gatifloxacin and the x axis represents the corresponding $\log_{10}$ MIC value of the ofloxacin of each tested *S.* Typhi isolate. Each point represents an individual *S.* Typhi strain (n = 297). The relationship is statistically significant ($p < 0.001$) using a Pearson test for linear correlation (*cor* = 0.812, CI 95 %).

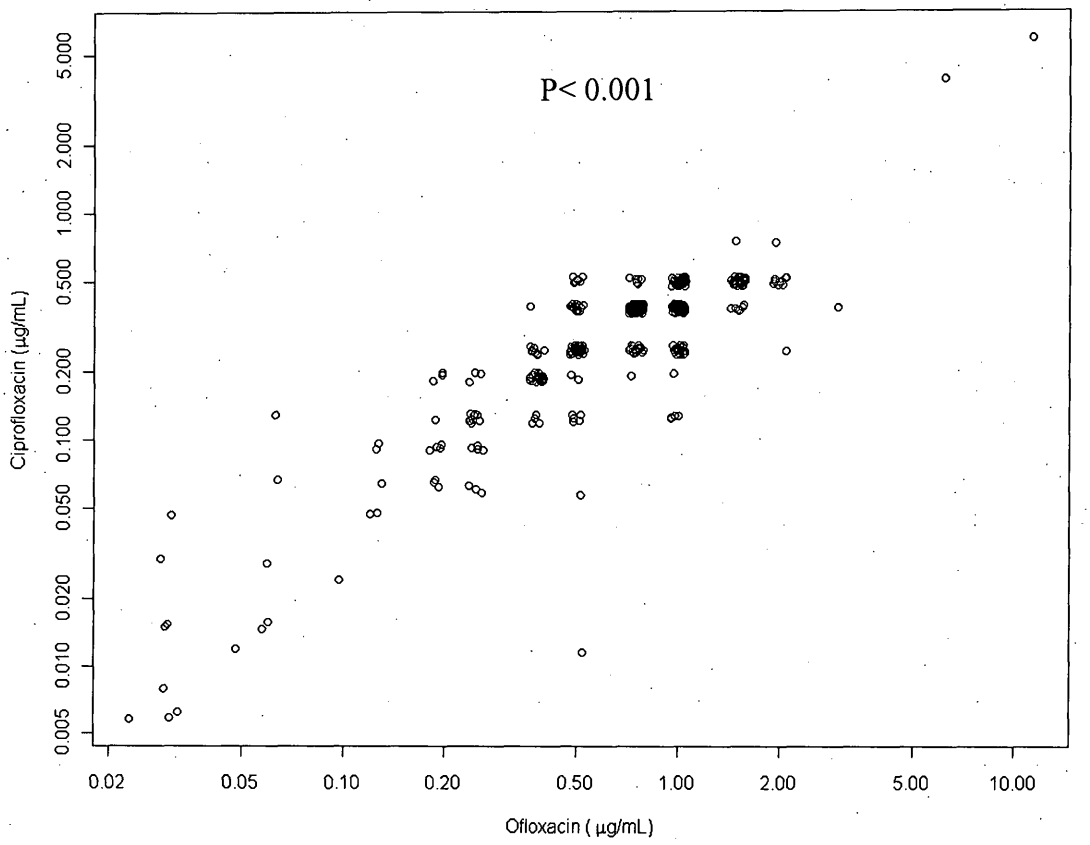


Figure 4.12 The relationship of ciprofloxacin and ofloxacin MICs of *S.* Typhi mutants and non - mutants from Asian countries

The y axis represents the $\log_{10}$ MIC value of ofloxacin and the x axis represents the corresponding $\log_{10}$ MIC value of the ciprofloxacin of each tested *S.* Typhi isolate. Each point represents an individual *S.* Typhi strain (n = 297). The relationship is statistically significant ($p < 0.001$) using a Pearson test for linear correlation ($cor = 0.867$, CI 95 %).

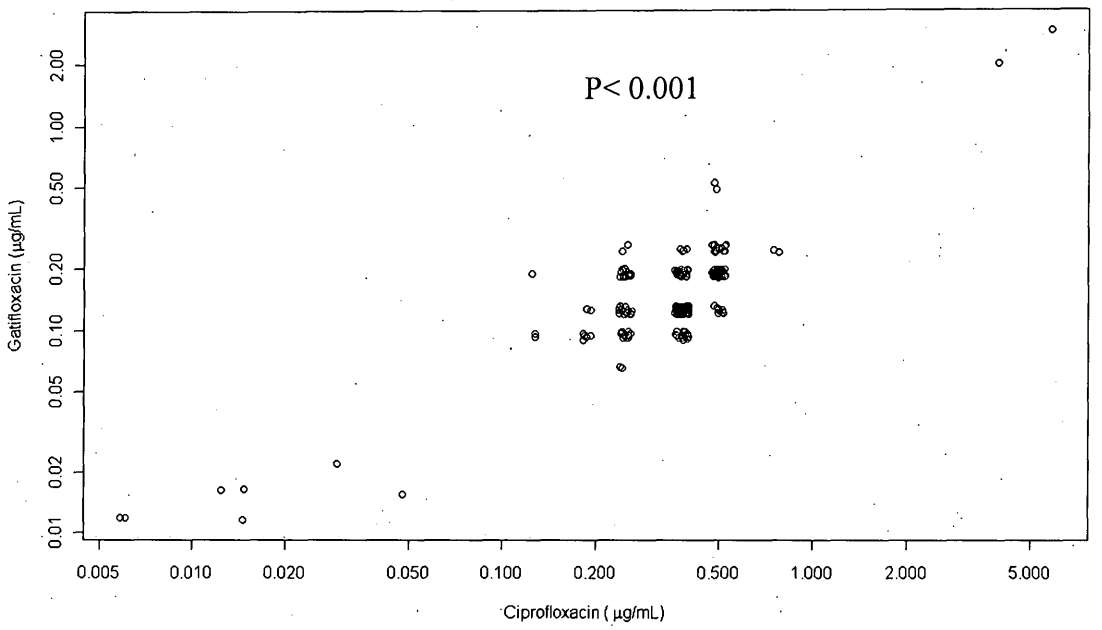


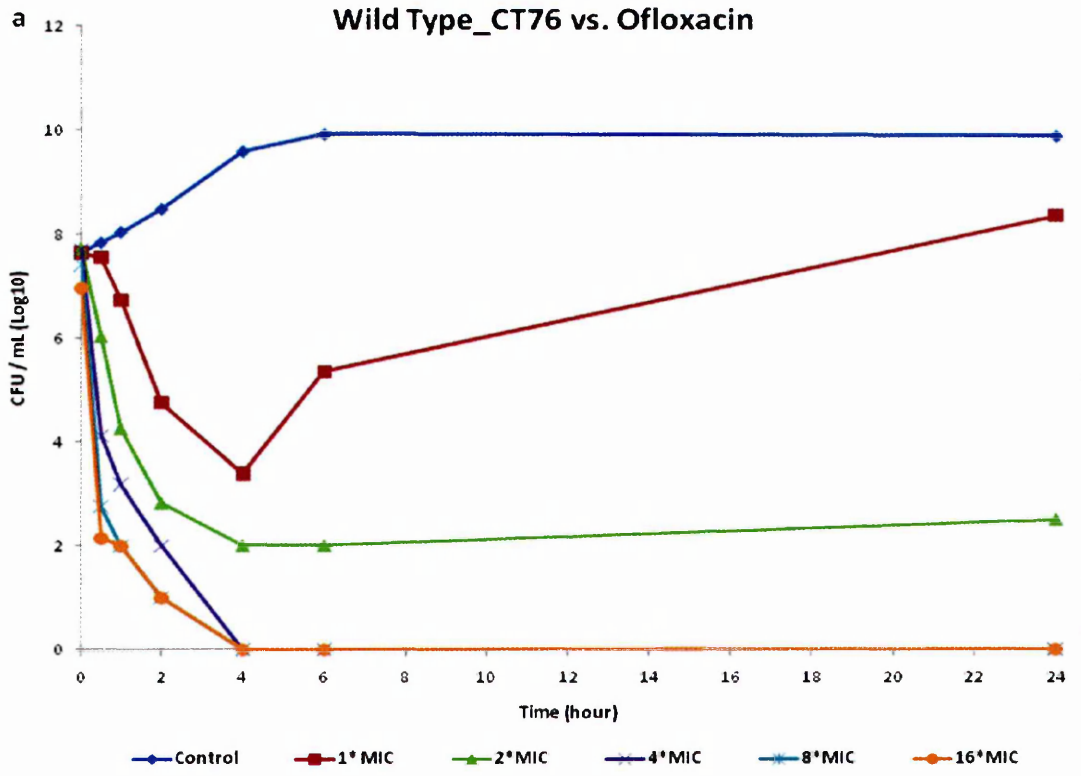
Figure 4.13 The relationship of ciprofloxacin and gatifloxacin MICs of *S.* Typhi mutants and non - mutants from Asian countries

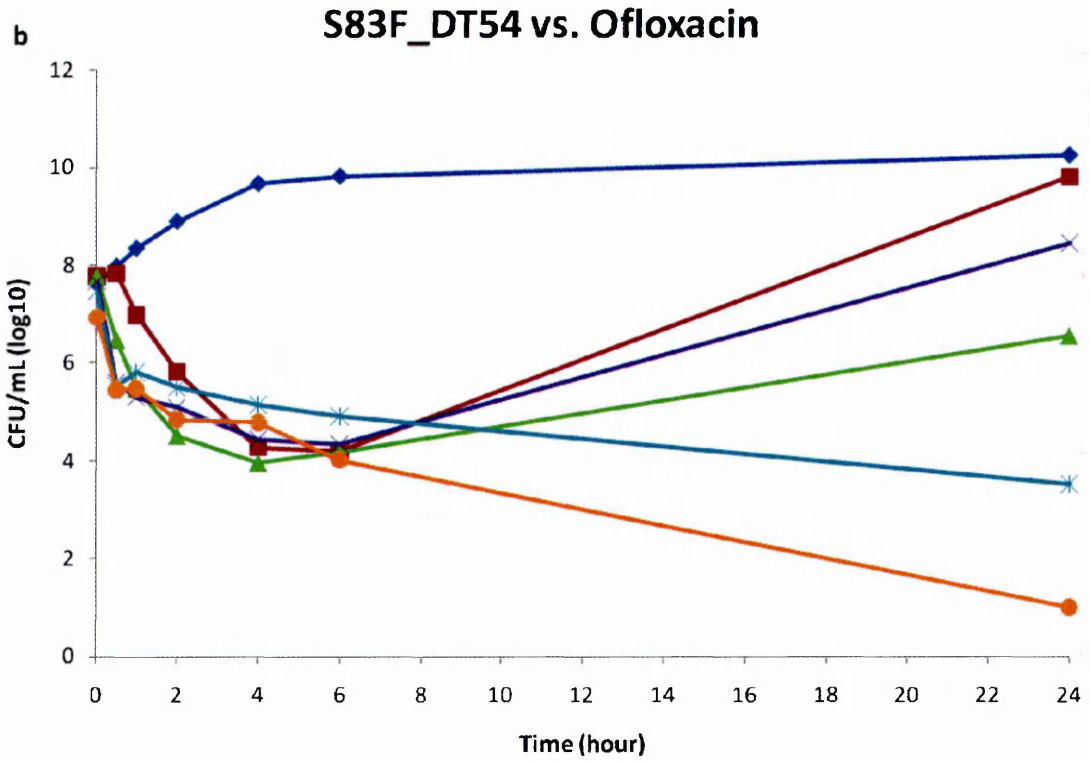
The y axis represents the $\log_{10}$ MIC value of gatifloxacin and the x axis represents the corresponding $\log_{10}$ MIC value of the ciprofloxacin of each tested *S.* Typhi isolate. Each point represents an individual *S.* Typhi strain (n = 297). The relationship is statistically significant ($p < 0.001$) using a Pearson test for linear correlation (*cor*= 0.816, CI 95 %).

4.2.4 *In vitro* time - kill analysis

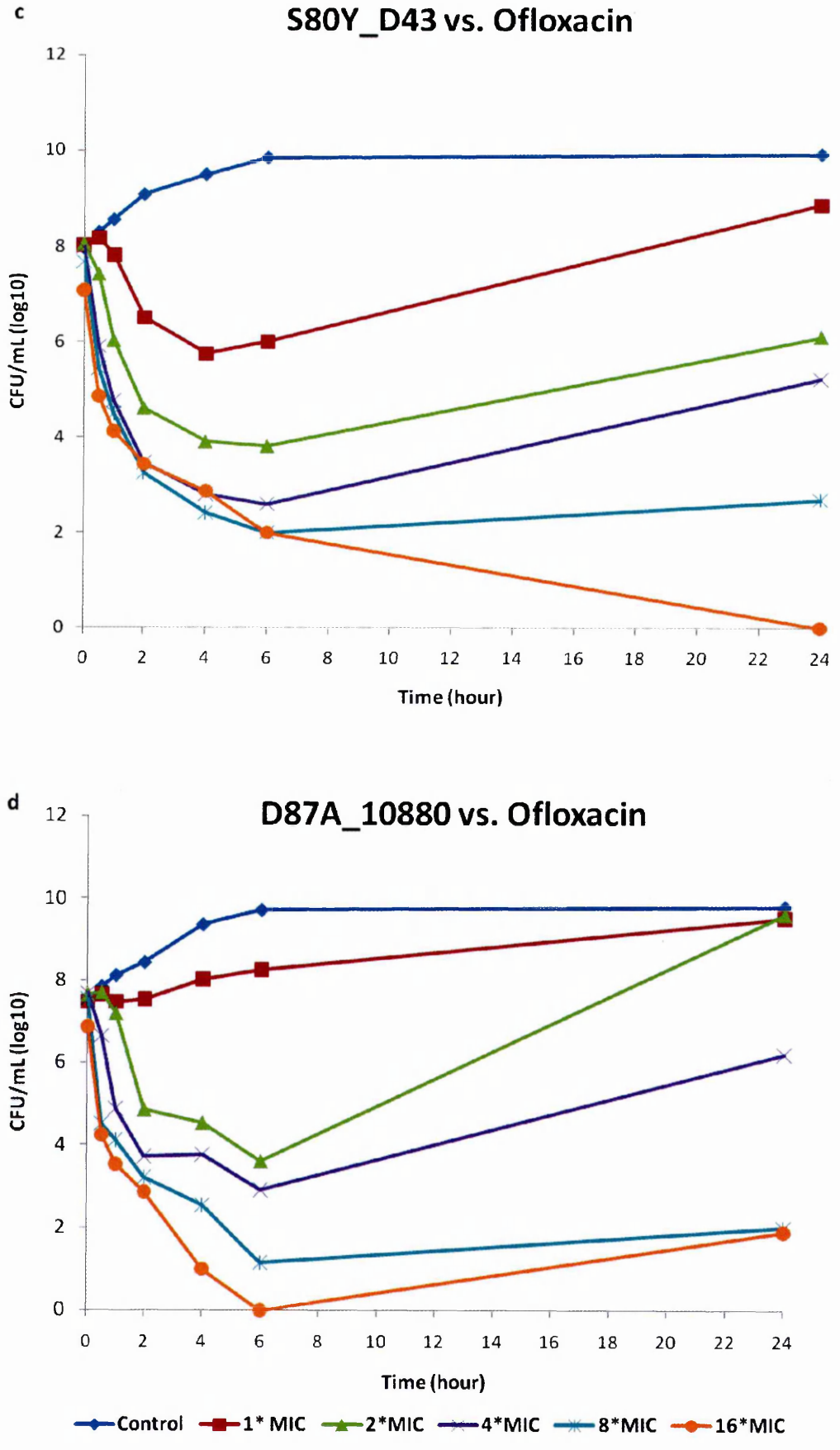
4.2.4.1 *In vitro* time - kill analysis of *S.* Typhi mutants to ofloxacin

MIC testing is useful for measuring resistance, however, it does not provide information on killing dose and how effective the antimicrobial is over a prolonged time period. One isolate that was representative of each of mutant groups (8 strains) and one non - mutant strain of *S.* Typhi was selected for *in vitro* time - kill experiments. The non - mutant strain was CT 76 (MIC 0.064 μg / mL to ofloxacin; MIC 0.008 μg / mL to gatifloxacin), and the mutant strains were DT54 (*gyrA* Ser 83 → Phe; MIC 1.0 μg / mL to ofloxacin; MIC 0.25 μg / mL to gatifloxacin), D 43 (Ser 83 → Tyr; MIC 0.5 μg / mL to ofloxacin; MIC 0.19 μg / mL to gatifloxacin), CT 144 (Asp 87 → Gly; MIC 1 μg / mL to ofloxacin; MIC 0.19 μg / mL to gatifloxacin), 10880 (Asp 87 → Ala; MIC 0.5 μg / mL to ofloxacin; MIC 0.032 μg / mL to gatifloxacin), A 54 (Asp 87 → Asn; MIC 1 μg / mL to ofloxacin; MIC 0.094 μg / mL to gatifloxacin), DT 18 (Ser 83 → Phe and Asp 87 → Gly; MIC 2.0 μg / mL to ofloxacin; MIC 0.5 μg / mL to gatifloxacin), AG 152 (Ser 83 → Phe and Asp 87 → Asn; MIC 3.0 μg / mL to ofloxacin; MIC 0.25 μg / mL to gatifloxacin) and C 2114 (Ser 83 → Phe, Asp 87 → Gly and S80I (*parC*); MIC 6.0 μg / mL to ofloxacin; MIC 2 μg / mL to gatifloxacin). The mean changes in $\log_{10}$ CFU / mL over 24 hours at 1, 2, 4, 8 and 16 times MIC to ofloxacin are presented in Figure 4.14.



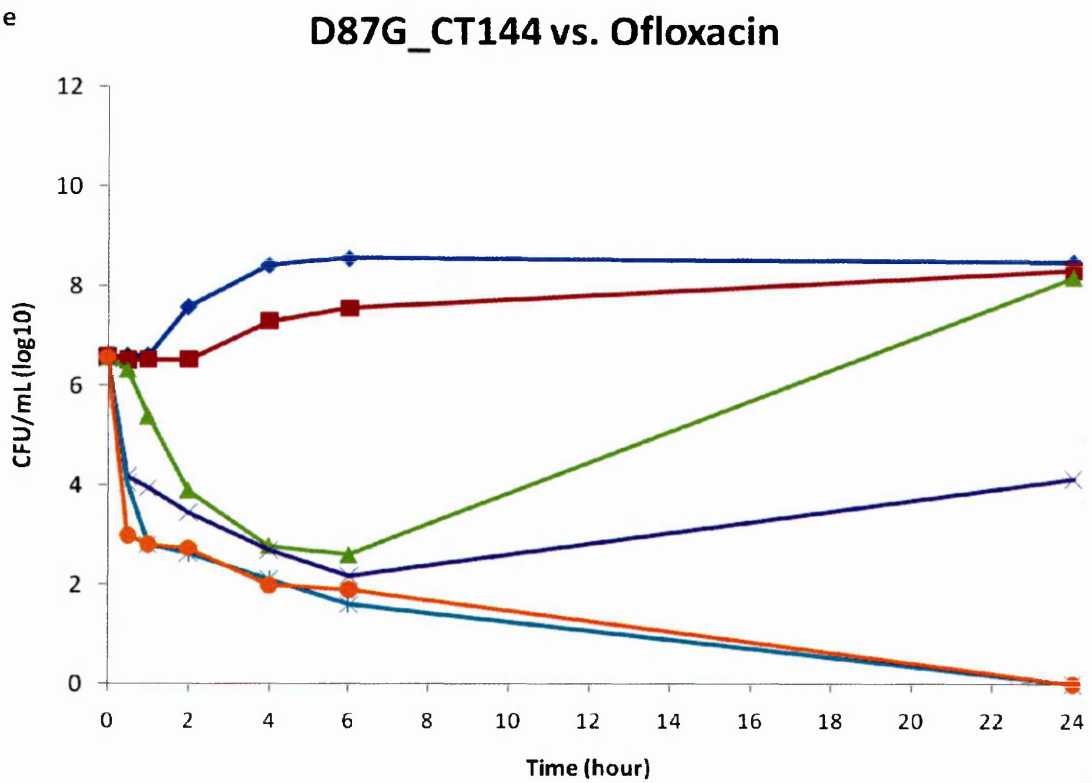


Wild type strain CT 76 (a) and *S.* Typhi DT 54 (Ser83→Phe) (b) were exposed to ofloxacin.

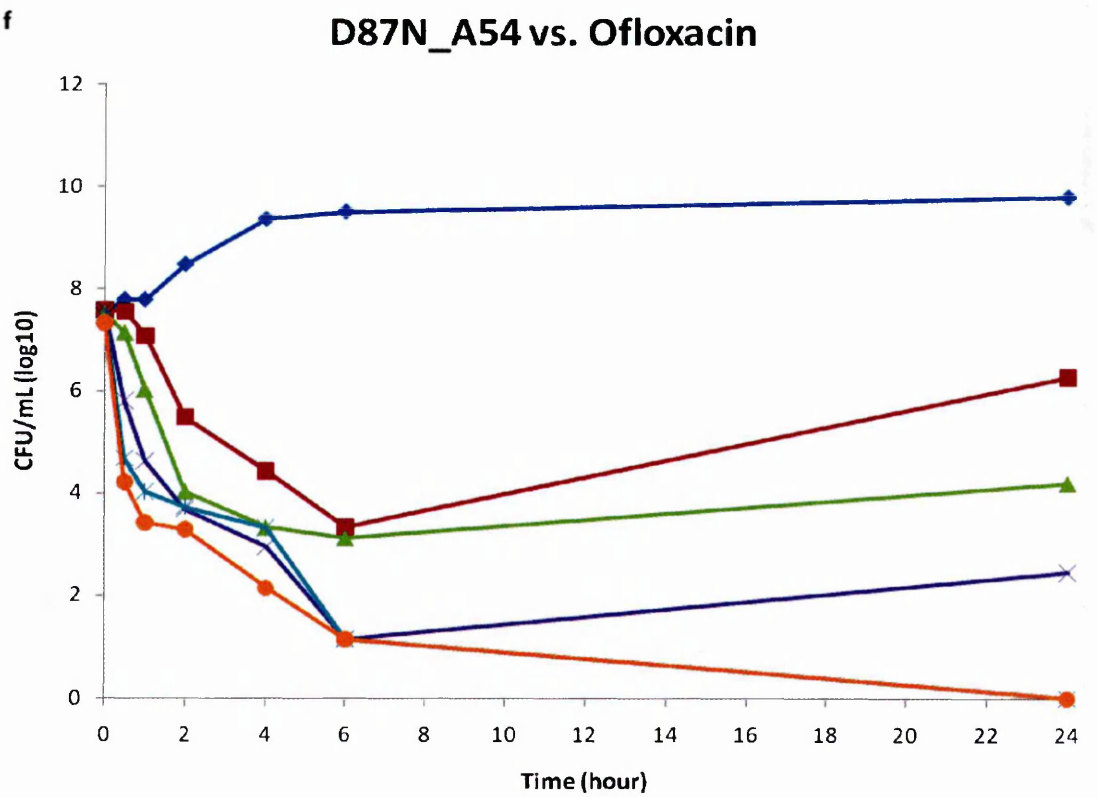


*S.*Typhi D 43 (Ser83→Tyr) (c) and *S.* Typhi 10880 (Asp87→Ala) (d) were exposed to ofloxacin.

e

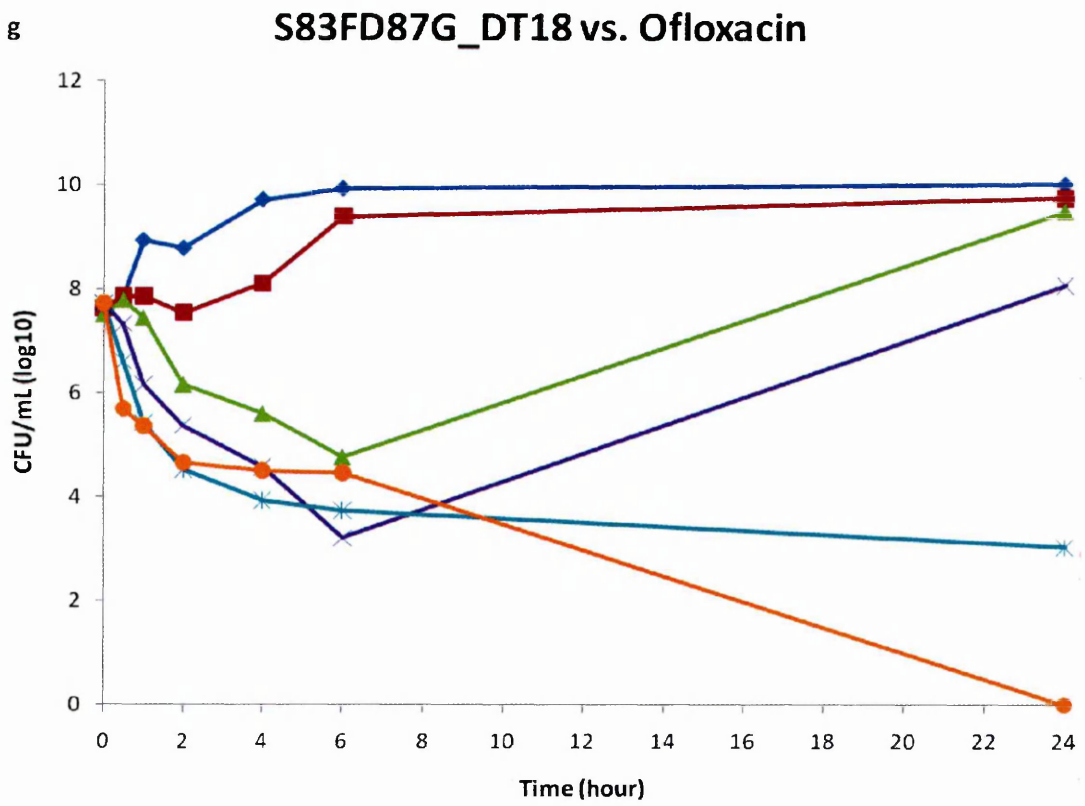


f

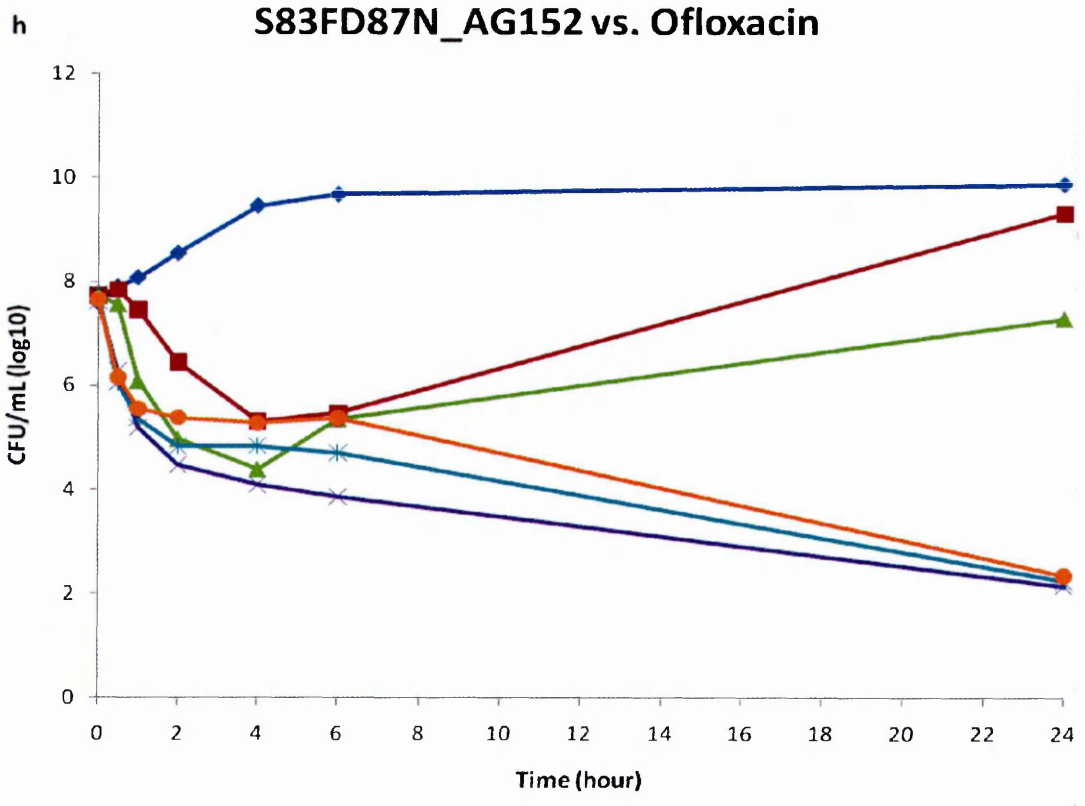


S. Typhi CT 144 (Asp87→Gly) (e) and A 54 (Asp87→Asn) (f) were exposed to ofloxacin.

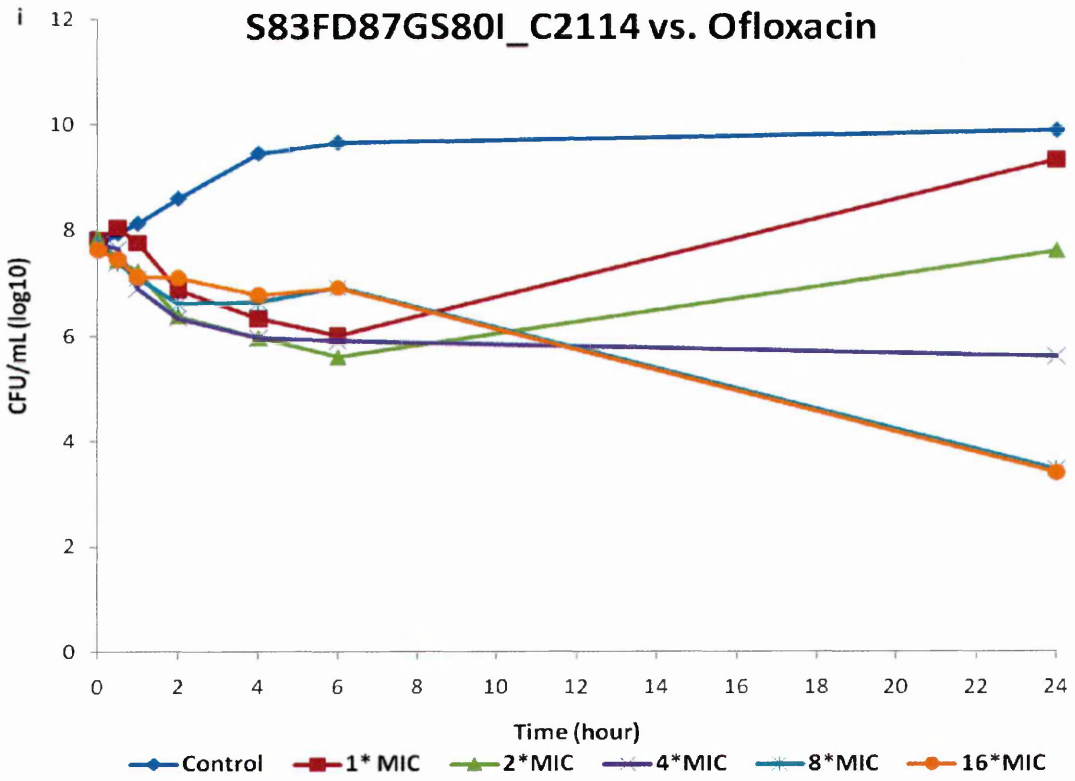
g



h



S. Typhi DT 18 (Ser83→Phe and Asp87→Gly) (g), *S.* Typhi AG 152 (Ser83→Phe and Asp87→Asn) (h) were exposed to ofloxacin.



S. Typhi C 2114 (Ser83→Phe and Asp87→Gly in *gyrA* and Ser80→Ile in *parC*) (i) was exposed to ofloxacin.

Figure 4.14 *In vitro* time - kill experiments of *S.* Typhi exposed to ofloxacin at concentrations of 1 x to 16 x MIC over 24 hours

In vitro experiments were performed in duplicate and results represent the mean of duplicate values, with error and standard deviation. The y axis represents the $\log_{10}$ value of *S.* Typhi CFU / mL. The x axis represents the time in hour. The *S.* Typhi growth was sampled at 6 time points, 30 minute, 1 hour, 2hours, 4 hours, 6 hours and 24 hours (section 2.6). Each line depicts an individual ofloxacin concentration in MIC and the MIC concentrations used against the organism tested. The subtitle of each diagram represents the mutation, the name of the mutant and the antimicrobial used.

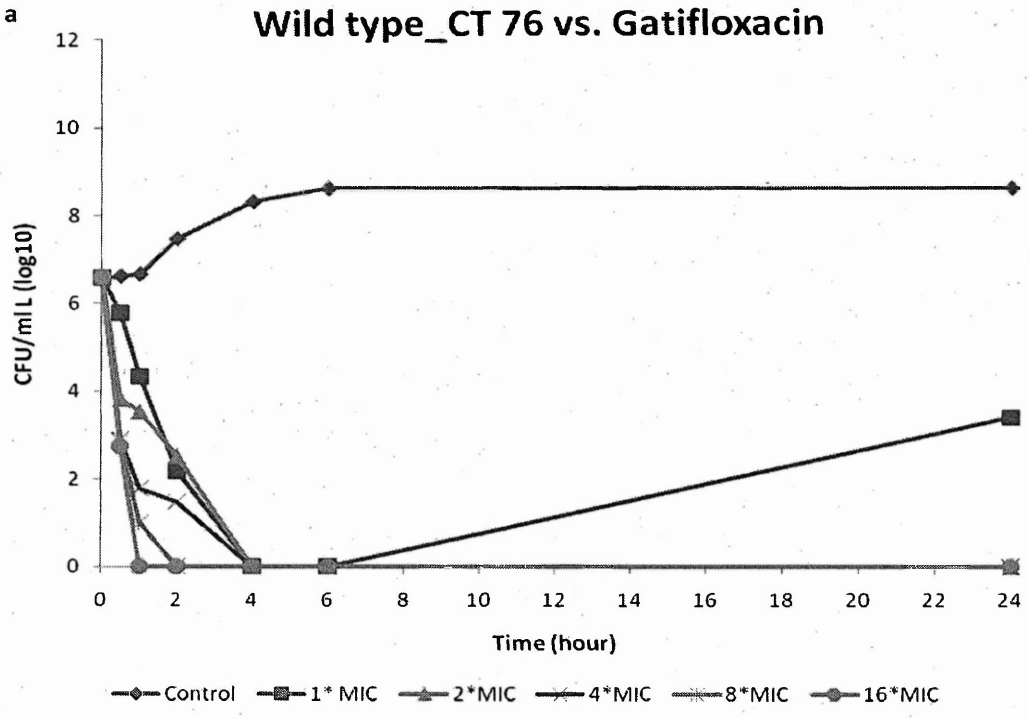
Ofloxacin showed rapid killing of the non - mutant, fluoroquinolone susceptible strain CT 76 at all concentrations, and we can also see concentration dependent killing (Figure 4.14a). There was a slight effect of MIC concentration of ofloxacin on killing when comparing the overall effect on the mutants S83F and S83Y (Figure 4. 15b and 4. 15c). One time the MIC of ofloxacin was more effective at killing the S83F mutant than the S83Y mutant. However, the S83F substitution demonstrated a higher level of resistance over time (in comparison to the S83Y mutant), with more organisms surviving at 2x

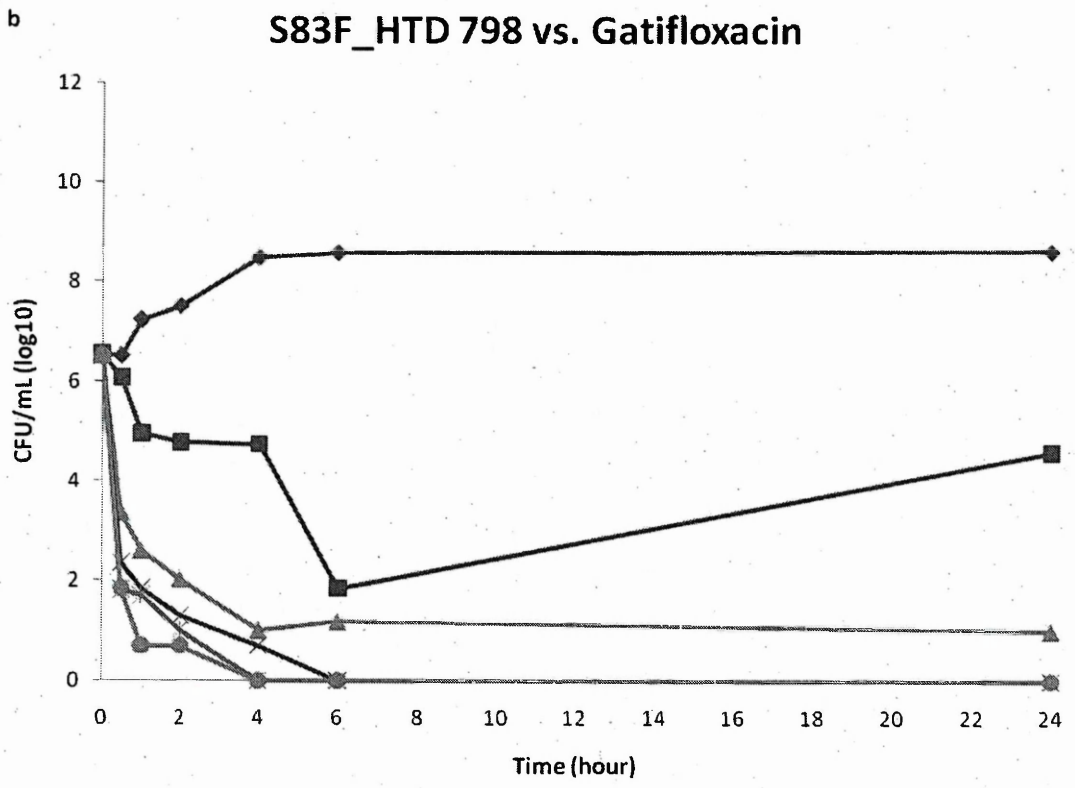
MIC after 6 hours of exposure (Figure 4. 15b and 4. 15c). The viable count of the S83F mutant reduced from 10^8 CFU / mL to 10^4 CFU / mL after six hours exposure at 2 x MIC, whilst the concentration of the S83Y mutant was reduced from 10^8 CFU / mL to 10^3 CFU / mL after the same exposure period. The majority of ofloxacin concentrations did not kill the strains with a single mutation at position 83, except after prolonged exposure at 16 x MIC (Figure 4. 15b and 4.14c). Time kill analysis of the position 87 substitutions showed that the D87G strain was more resistant to ofloxacin than those strains with the substitution of Alanine or Asparagine for Aspartic acid at the same position. The number of viable D87G and D87A mutant bacteria decreased from 10^7 CFU / mL to 10^2 CFU / mL after 6 hours with 16 x ofloxacin MIC. Whilst the D87N strain decreased from 10^7 CFU / mL to 10 CFU / mL over the same exposure duration and at the same concentration of ofloxacin (Figure 4.14d and 4.14e).

Time kill analysis comparison of the double *gyrA* gene mutations, DT 18 (S83F & D87G) and AG 152 (S83F & D87N), demonstrated that the viable counts of bacteria of both mutants decreased in the first 6 hours of exposure to 2x MIC to16x MIC and 1x MIC to 16x MIC of ofloxacin, respectively (Figure 4.14g and 4.14h). After six hours of exposure to ofloxacin the bacterial counts of the S83F D87G strains recovered to 10^7 CFU / mL while the bacterial count of the S83F D87N mutation decreased to 10^2 CFU / mL after 24 hours (Figure 4.14g and 4.14h). Complete killing of both the double mutants could not be achieved at any MIC in the experiment over a 24 hour period. There was only limited bactericidal activity of ofloxacin against the triple mutant in comparison to the control. This lack of bactericidal activity was seen with all ofloxacin concentrations over a 24 hour period in strain C 2114 with the substitution S83F D87G in *gyrA* and S80I in *parC* gene (Figure 4.14i).

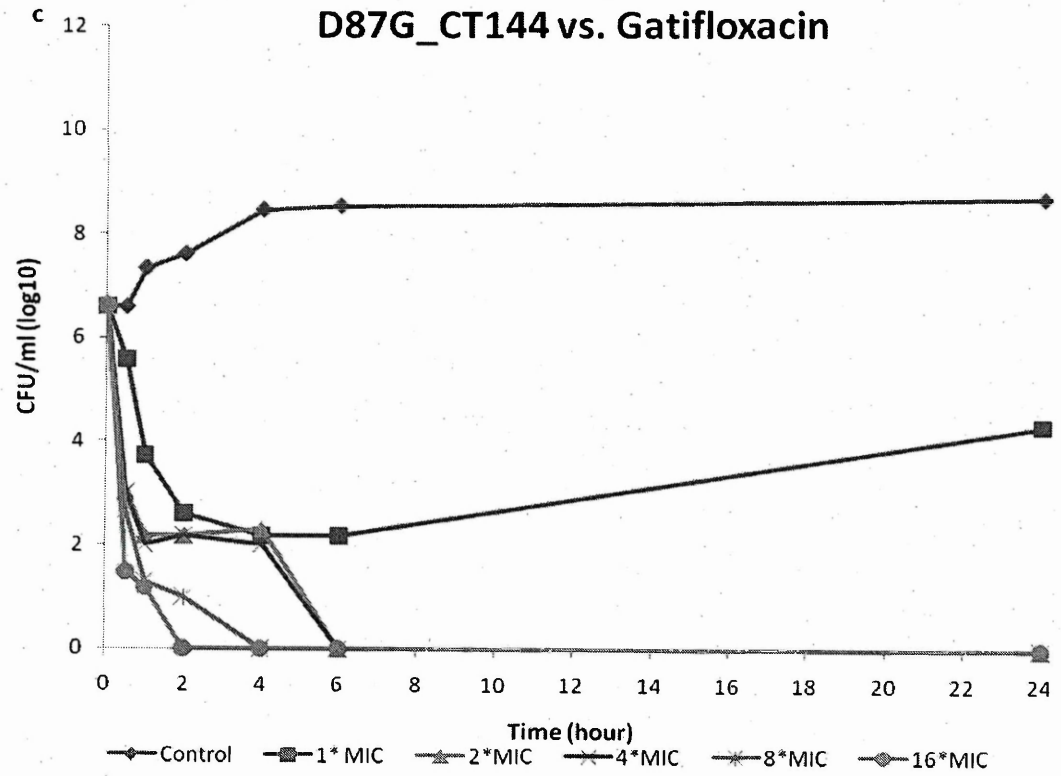
4.2.4.2 *In vitro* time - kill analysis of *S.* Typhi mutants with gatifloxacin

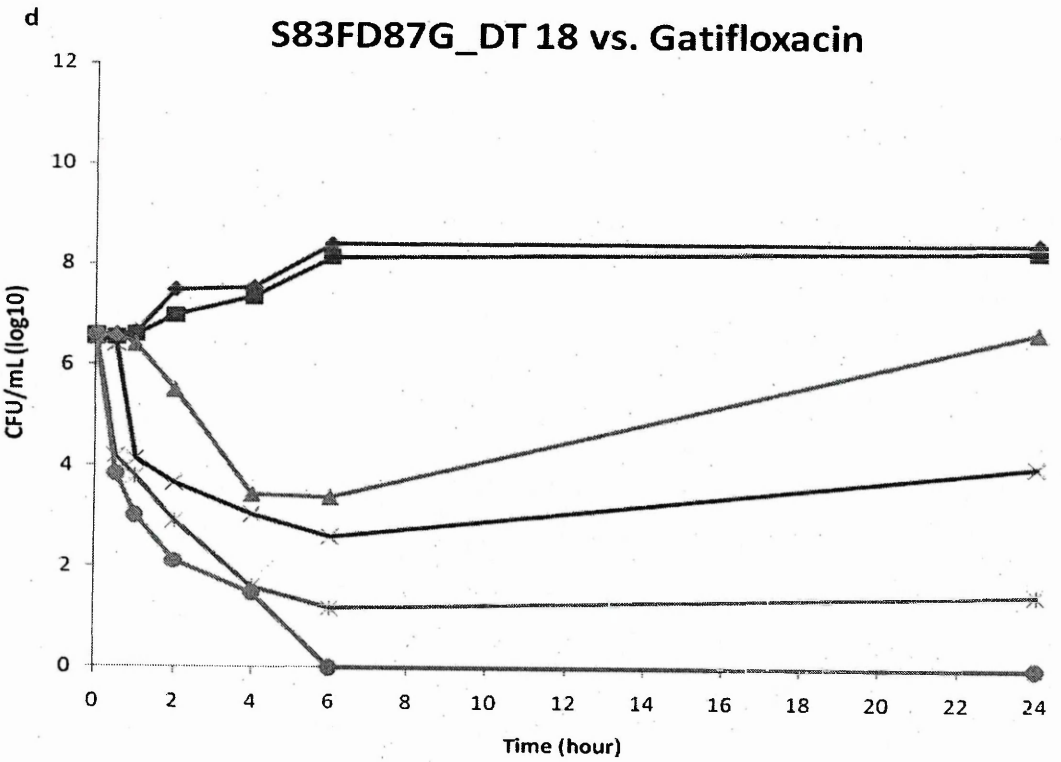
A subset of representative mutations and the non - mutant strain were subjected to a time - kill experiment to gatifloxacin. These strains were, CT 76, HTD 798 (Ser83 → Phe; MIC 1 μg / mL to ofloxacin; MIC 0.125 μg / mL to gatifloxacin), CT 144, DT 18 and C 2114. The mean changes in $\log_{10}$ CFU / mL with exposure to differing concentrations of gatifloxacin over a 24 hour period are presented in Figure 4.15.



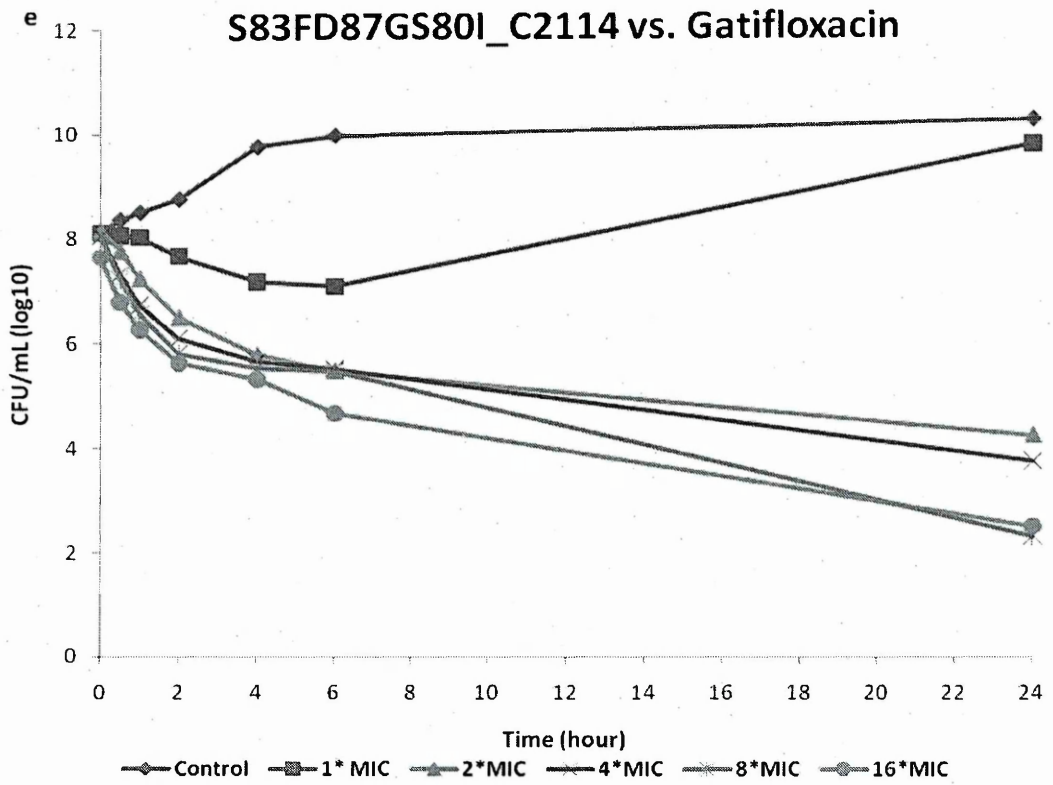


Wild type *S.* Typhi strains CT 76 (a) and the mutant HTD 798 (Ser83→Phe) (b) were exposed to gatiloxacin.





S. Typhi CT 144 (Asp87→Gly) (c) DT 18 (Ser83→Phe and Asp87→Gly) (d) were exposed to gatifloxacin.



S. Typhi C 2114 ((Ser83→Phe, Asp87→Gly in *gyrA* and Ser80→Ile in *parC*) (e) was exposed to gatifloxacin.

Figure 4.15 *In vitro* time - kill experiments of *S.* Typhi strains exposed to gatifloxacin

In vitro experiments were performed in duplicate and results represent the mean of duplicate values, with error and standard deviation. The y axis represents the $\log_{10}$ value of *S.* Typhi CFU / mL. The x axis represents the time in hour. The *S.* Typhi growth was sampled at six time points, 30 minute, 1 hour, 2hours, 4 hours, 6 hours and 24 hours (section 2.6). Each line depicts an individual gatifloxacin concentration in MIC and the MIC concentrations used against the organism tested. The subtitle of each diagram represents mutation type, the name of the mutant and antimicrobial used.

Gatifloxacin at 4 x MIC had the largest effect on decreasing the viable number of *S.* Typhi strains CT 76, HTD 798 and CT 144 (Figure 4.15 a,b,c) in the first 30 minutes of exposure and demonstrated total killing after 6 hours. Gatifloxacin rapidly killed strain CT 76 after 4 hours, this occurred independently of the concentration of the antimicrobial (Figure 4.15a). The survival of the HTD 798 (S83F mutant) was only observed in 1x MIC of gatifloxacin yet the viable bacterial count decreased from 10^7 CFU / mL to 10^5 CFU / mL (Figure 4.15b). CT 144 (D87G mutant) was more susceptible to gatifloxacin than the HTD 798 (S83F mutant) mutant since the viable bacterial concentrations were reduced from 10^7 CFU / mL to 10^3 CFU / mL after 4 hours at all concentrations of gatifloxacin (Figure 4.15c).

Viable counts of *S.* Typhi DT 18 (S83F and D87G mutant) decreased after 4 hours followed by some re-growth, higher concentrations (8x and 16x MIC) of gatifloxacin showed a more pronounced bactericidal effect against this double mutant (Figure 4.15d). There was no total killing activity of gatifloxacin against the triple mutation *S.* Typhi C 2114 with a double *gyrA* substitution (S83F D87G) and a single substitution in the *parC* gene (S80I) (Figure 4.15e).

The mean *S.*Typhi inoculum calculated from all mutants against 4 times MIC of ofloxacin and gatifloxacin at the starts of the killing assay (Time 0) were 5.26 x 10^7 CFU/mL (3.30 x 10^7 – 7.20 x 10^7, CI 95%) and 2.99 x 10^7 CFU/mL (1.23 x 10^7 – 7.20 x 10^7, CI 95%), respectively. Therefore, the results calculated after 4 hours exposure to 4 times MIC of ofloxacin were dependant on the experimental behavoiur of the individual mutants and not the initial starting innoculum.

Time - kill analysis demonstrated that an increase in survival ability significantly correlated with the number of mutations at 4x MIC of ofloxacin at 4 hours of exposure (p linear trend <0.001) (Figure 4. 16). However, the number of mutations did not statistically influence the concentration of bacteria in 4x MIC of gatifloxacin at 4 hours (p linear trend was 0.002, the linear correlation is significant if p linear trend is less than 0.001) (Figure 4.17).

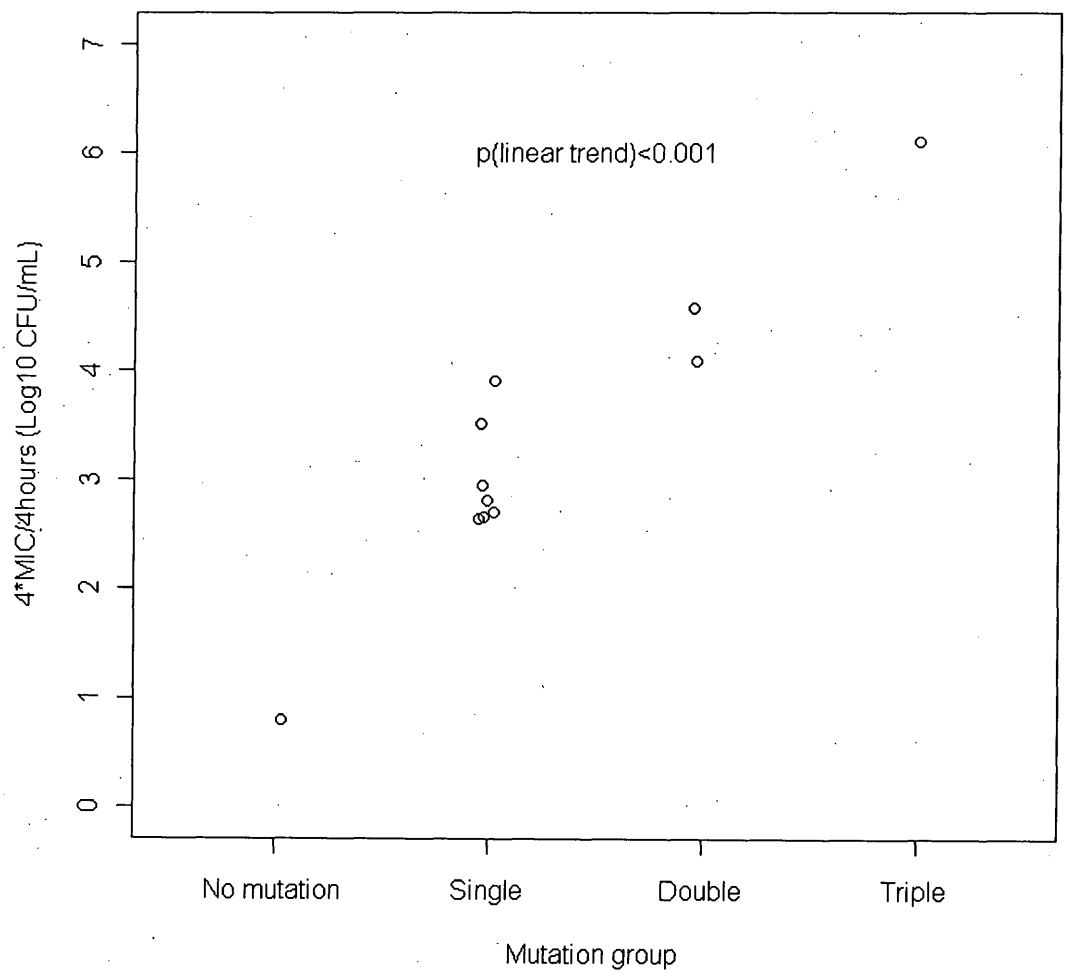


Figure 4.16 The killing rate relationship with ofloxacin to different *S.* Typhi mutants and non - mutants from Asian countries

The y axis represents the $\log_{10}$ CFU/mL of *S.* Typhi strains after 4 hours of exposure to 4x MIC of ofloxacin. The x axis represents 4 *S.* Typhi groups including non - mutant, single, double and triple mutation groups. Each point represents an individual *S.* Typhi strain (n = 11). The relationship is statistically significant with p linear trend < 0.001 using linear regression test.

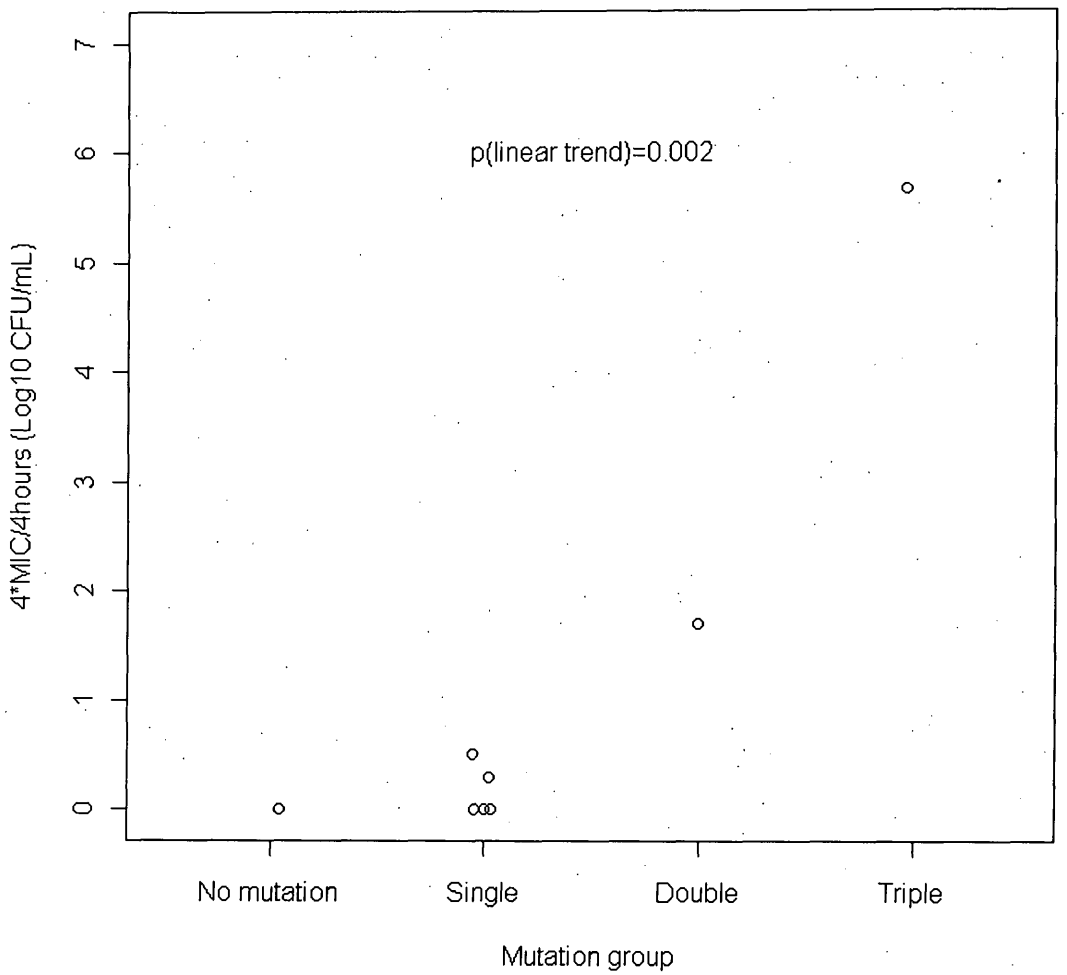


Figure 4.17 The killing rate relationship with gatifloxacin to different *S.* Typhi mutants and non - mutants from Asian countries

The y axis represents the $\log_{10}$ CFU/mL of *S.* Typhi strains after 4 hours of exposure to 4x MIC of ofloxacin. The x axis represents 4 *S.* Typhi groups including non - mutant, single, double and triple mutation groups. Each point represents mean of $\log_{10}$CFU / mL of an individual *S.* Typhi strain (n = 8). *p* linear trend was 0.002 using linear regression test.

4.3 Discussion

This is the first extensive, systemic molecular characterization of a large number of nalidixic acid resistant and reduced susceptibility to fluoroquinolones *S.* Typhi isolates across Asian countries. All *S.* Typhi isolates (n = 462) which were resistant to nalidixic acid and had reduced susceptibility to fluoroquinolones haboured at least one mutation in the *gyrA* gene; our findings were supported by previous studies looking at fluoroquinolone resistance in the *Salmonella* [147,162,199,226].

The majority of the mutations in *gyrA* and *parC* genes caused resistance to nalidixic acid and reduced susceptibility to ofloxacin, ciprofloxacin and gatifloxacin. Whilst statistical calculations demonstrated significant differences in MIC levels between mutant and non - mutant groups, all the MIC values were still lower than the MIC corresponding to resistance according to the current CLSI guidelines [141]. We originally observed only two *S.* Typhi isolates in India which were completely resistant to ofloxacin and ciprofloxacin and these strains had only intermediate resistance to gatifloxacin, thus demonstrating differing biological activities of the various classes of fluoroquinolones.

Substitutions at position 83 of the *gyrA* were the most common mutation detected in the Vietnamese and Indian clinical isolates (n = 405); the substitution of Phenylalanine for Serine at codon 83 in the *gyrA* gene occurred mostly in Viet Nam isolates. The substitution of Tyrosine for Serine was only detected within Indian isolates. Investigating the mechanism responsible for nalidixic acid resistance in seven *S.* Typhi isolates in India, Capoor did not find any strains with a substitution at position 83 in the *gyrA* gene [190]. Double and triple mutations were also found to be uncommon in this Indian study. Whilst the triple amino acid substitution (Ser83 → Phe, Asp87 → Gly in

gyrA and Ser80→Ile in *parC*) genes in our study confirmed the result of Gaind's findings [191],Capoor and Dimitrov found that ciprofloxacin resistant *S.* Typhi strains had a different mutation in *gyrA* gene to those detected here [190,213]. A similar study conducted in twenty-five and six strains of *S.* Typhi which had decreased susceptibility to ciprofloxacin and were ciprofloxacin susceptible, respectively, attempting to identify the genetic basis of nalidixic acid resistant and reduced susceptibility to fluoroquinolones *S.* Typhi did not find any mutation in the QRDR of the *gyrB*, *parC* and *parE* g enes [226]. In other studies conducted with *S.* Typhi isolates in India, Kuwait, mutations were also identified outside the QRDR of the *gyrA*, *parC* genes [213].

The basis of a strong association between fluoroquinolone resistance and *gyrA* mutations which are frequently observed at codon 83 and codon 87 is a clustering of these mutations around the active site at location Tyr 122 in the protein structure [83], this is known to be the binding site of fluoroquinolones (Figure 4.18). Consequently, mutations at this location change the structure and the electrical charge of the enzyme active site, ultimately, the bactericidal activity of fluoroquinolones is hampered due to an inhibition of binding activity of chemical to the DNA gyrase [227].

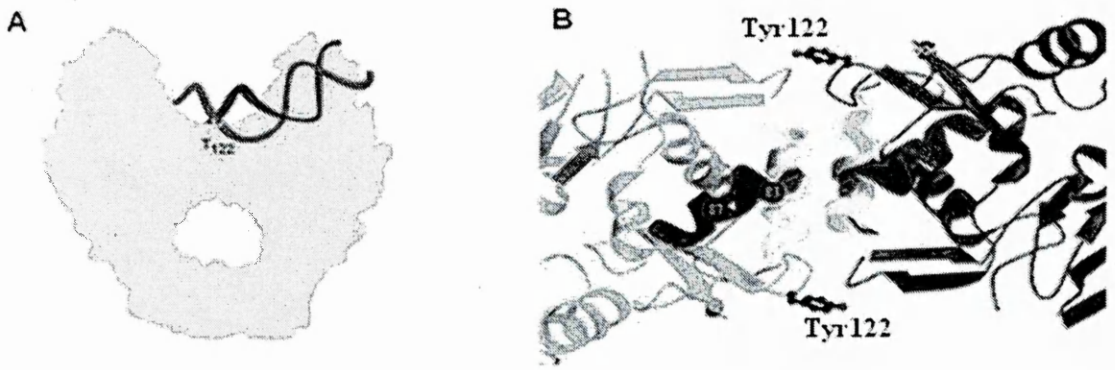


Figure 4.18 The A subunits of gyrase enzyme of gram negative bacteria

The figure was adapted from Cabral *et al.* [228]. (**A**) The light blue shape depicts the subunit A of gyrase, enzyme active site Tyrosine 122 is presented as yellow star and double strands DNA are depicted as red and green ribbons. (**B**) The crystal structure of the active site region of gyrase. Helix–turn–helix motifs are shown in yellow and red, some secondary structure elements are indicated in light green and dark blue. Two commonly mutated loci, Serine 83 and Aspartic acid 87, are shown as numbered black spheres.

Although plasmid mediated quinolone resistance has been widely observed in other gram negative bacteria, and occasionally defined in ciprofloxacin resistant *S.* Typhi [191], we did not detect any *qnrA* and *qnrS* genes in the collection across Asian countries. Our work is supported by a study conducted in 499 *Salmonella* strains in France. In this study, none of *qnrA*, *qnrB* and *qnrS* genes were detected in all quinolone resistant *Salmonella* strains including 13 *S.* Typhimurium strains, 11 *S.* Enteritidis strains and 63 *S.* Hadar strains except one *S.* Concord strain which haboured ESBL encoded genes ($bla_{CTX\text{-}M\text{-}15}$) also carried the *qnrA1* gene [229]. This is consistent with the theory that *S.* Typhi and other *Salmonella* species maybe genetically isolated and organisms have limited opportunity of uptaking genes via conjugation or bacteriophage mediated transduction. In order to observe the resistance mechanisms which may be related to reducing drug uptake or enhancing efflux pump activity, we additionally screened *S.* Typhi strains for organic solvent resistance using hexane and cyclohexane. The screening results showed no cyclohexane resistance in the *S.* Typhi strains tested. However, further experiments using molecular approaches could be designed to

investigate any genomic variations or gene expression changes that may aid understanding of resistance mechanisms. The new application of DNA pyrosequencing in this study permitted a level of high throughput sequencing to detect mutations in the QRDR of *gyrA* gene in a large number of *S.* Typhi strains. However, for extensive investigation of mutations, conventional DNA sequencing is still the gold standard. There may be an additional need to extend the sequencing of the QRDR regions of genes encoding gyrases and topoisomerases VI in the study of fluoroquinolone resistance in *S.* Typhi since recent reports of the presence of mutations related to resistant phenotype that lie outside the QRDR region [206].

To understand the correlation between mutations and the MICs of fluoroquinolones, we analyzed the mutations in the associations with ofloxacin, ciprofloxacin and gatifloxacin which are the current antimicrobial agents used clinically in typhoid treatment. Most *S.* Typhi mutants had high MICs to nalidixic acid, demonstrating a significant increase with respect to the non - mutant isolates. The MIC value comparisons between the different groups of mutants and the non - mutants to ofloxacin, ciprofloxacin and gatifloxacin demonstrated statistically significant variation. The MIC comparison among mutation groups showed the triple mutants had the highest MIC level, the next highest was the double mutants and the lowest increase was the single mutants. We found a linear relationship in MIC values among the mutants between ofloxacin and ciprofloxacin to gatifloxacin. This linear relationship was the most prominent with respect to the increase in MIC to ofloxacin and ciprofloxacin. This is probably due to the similarity in the action mechanism of these antimicrobials to *S.* Typhi isolates and that these chemicals differ from the antimicrobial activities (active site target) of gatifloxacin. Gatifloxacin has different capabilities in comparison to other

fluoroquinolones as it is known to equally affect both the gyrase of *Enterobacteriaceae* and topoisomerase VI in gram-positive bacteria [230].

We found that the MIC levels to fluoroquinolones showed a significant difference between the mutants and non - mutants and there was a variation in the MIC level amongst the mutants. However, the bactericidal effects of these antimicrobial agents to strains with differing mutations were not directly comparable by MIC comparison alone. To understand the bactericidal related effect of *gyrA* and *parC* mutations of *S.* Typhi strains, we applied the time - kill experiments to evaluate the resistance phenotype of the different *S.* Typhi mutants detected in Asian clinical collection. Historically, time - kill curves have been used in evaluating the efficacy of new drugs. Time - kill curves are rarely used to guide therapeutic doses of an antimicrobial. Both concentration-dependent and time-dependent bactericidal activities of antimicrobial agents can be studied using time - kill analysis [231].

Time - kill analysis demonstrated that a significant increase in survival correlated with the number of mutations at 4x MIC of ofloxacin but was not associated with the number of mutations at the same concentration of gatifloxacin within 4 hours of exposure. This means that combinations of mutations in *gyrA* and *parC* gene influence the resistance to ofloxacin, but that mutation combination seems to not affect the efficacy of gatifloxacin, however, the triple mutant was an exception. Thus, the time - kill experiments suggest that the choice of the fluoroquinolones and the dose used for the treatment of *S.* Typhi may be critical and underlines clearly that resistance measured purely by MIC may not be the best marker of dosage. Continued use of the older generation fluoroquinolones (ofloxacin and ciprofloxacin) may encourage the persistence of resistant isolates and lead to the development of new mutations which

might compromise the efficacy of the newer generation treatments. Gatifloxacin (and potentially other newer generation fluoroquinolones) may prove a better choice for use in typhoid fever as it showed better response in the time - kill assays and may work more efficiently than other fluoroquinolones at a corresponding MIC. This provides a clear rationale for the clinical assessment of these drugs in randomized controlled trials in typhoid fever. If these *in vitro* data are supported by clinical results then newer generation fluoroquinolones should be recommended for the treatment of typhoid fever instead of ciprofloxacin and ofloxacin.

5. Reconstruction of fluoroquinolone resistant mutations in *gyrA* and *parC* genes in *S.* Typhi

5.1 Introduction

Fluoroquinolone resistant phenotypes in *Salmonella* species have been demonstrated here to be associated with mutations in topoisomerase genes which encode gyrase and topoisomerase IV enzymes [219,223,226]. In chapter 4, during DNA gyrase screening, the substitutions of an amino acid at Ser83 and Asp87 in the *gyrA* and the substitutions of the amino acid at Ser80 in the *parC* gene of *S.* Typhi strains were the most commonly found mutations. These substitutions have been observed both in clinical isolates [162,226,232-234] and when *Salmonella* isolates have been exposed to fluoroquinolones under experimental conditions [220].

Our work demonstrated the overall relationship and the distribution of mutations in the *parC* and *gyrA* genes with respect to fluoroquinolone resistance in clinical isolates. Despite the association of *gyrA* and *parC* mutations with fluoroquinolone resistance, the real impact of fluoroquinolone resistant mutants has not been investigated in either clinical isolates or *in vitro* selected *S.* Typhi strains. We know that from data presented here that fluoroquinolone resistant *S.* Typhi has become common and we hypothesize that these strains have disseminated across Asia, responding to the common use of these chemicals. In other words, these organisms are now under a strong natural selective pressure, as demonstrated by SNP selection by Roumagnac *et al.* [71]. Additionally, due to most of the work being performed on clinical isolates, the overall effect of such mutations is difficult to assess. Bacterial chromosomes are complex and along with mutations associated with antimicrobial resistance there may be compensatory mechanisms, in the form of regulators or other mutation sites. Therefore, to assess the overall effect, albeit in a laboratory setting, the strains used for such comparisons

should be isogenic. As a result of introducing point mutations into a known genetic background of an attenuated *S.* Typhi strain, Turner *et al.* (2006) showed that the single amino acid substitution in *gyrA* was sufficient for resistance to the nalidixic acid but remained susceptible to fluoroquinolones. Full resistance to fluoroquinolones required more than one mutation in *gyrA* and a mutation in *parC* genes [131].

Allelic exchange methodology is recognized as the method of choice for specific reconstructive mutagenesis in bacterial genes [235]. The allelic exchange method has been frequently used in bacteria to generate knockout genes, to carry out phenotypic analysis and to learn about their function [235]. This method is more suitable than other systems for generating individual nucleotide changes as the target site is easily controllable and does not leave a genetic "scar". Other advantages of such vectors are, they do not damage the bacterial chromosome (in other locations) and they permit a combination of several mutations in the same genetic background [236]. Suicide plasmids are so called as they usually contain the *sacB* gene, which leads to the death of the transformed bacteria when they are plated in the presence of sucrose [237]. The first application of the allelic exchange method using the suicide vector pJCB12 (as used here) constructed deletion mutations in an *E. coli* vaccine candidate [118], followed by secondary study which introduced fluoroquinolone resistance mutations into the topoisomerases of *S.* Typhi strains [131]. Introducing a mutation into bacterial cells using allelic exchange methodology involves two steps. Firstly, the entire plasmid is integrated into the chromosome by a single-crossover between the homologous region, producing a chromosomal duplication. Secondly, the chromosomal duplication is segregated by homologous recombination between the flanking direct repeats. As a result of recombination, one copy of the gene is left on the chromosome, either the wild

type copy or the mutant copy (Figure 5.1). Finally, the integrated plasmid is de-selected using the *sacB* expression system [238].

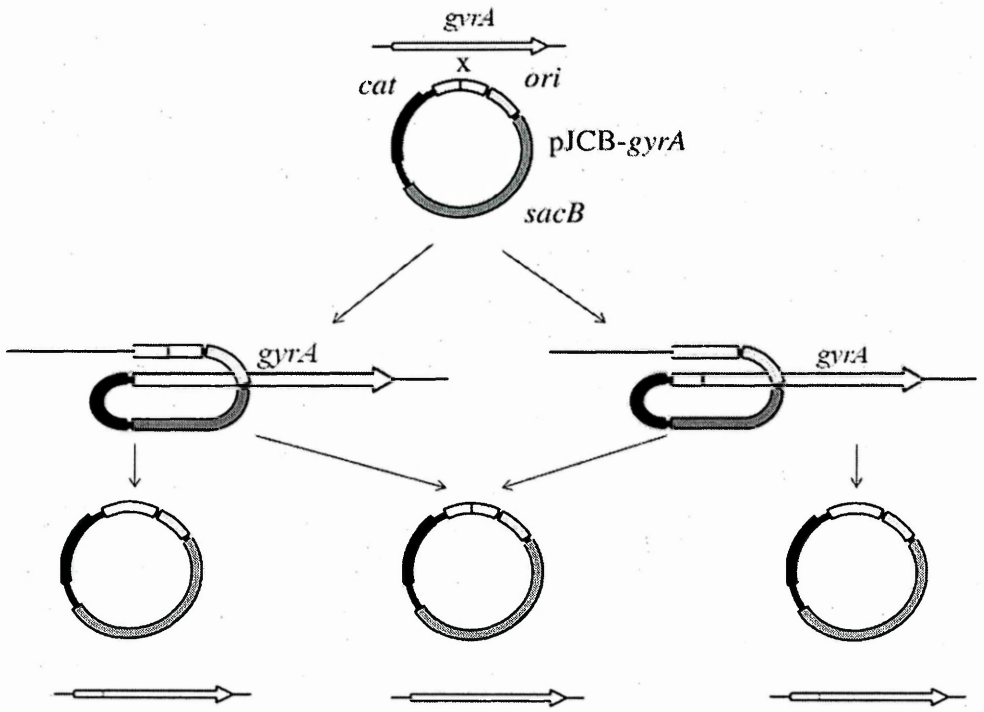


Figure 5.1 Allelic exchange methodology applying for the construction of a mutation in the *gyrA* locus.

The open boxes represent *gyrA* fragments and the open arrows represent the *gyrA* gene. The line through these represents the point mutation. The other loci of the pJCB12-*gyrA* are represented by different shaded boxes. Derivatives with pJCB12-*gyrA* incorporated at *gyrA* occurred depending on which side of the point mutation. The cross-over event occurs during homologous recombination. Likewise, following selection for recombinants that have lost the pJCB12-*gyrA* DNA, both derivatives generate resistant mutants or susceptible revertants (adapted from Turner *et al*) [118].

Although gyrase and topoisomerase IV mutations have been demonstrated to be associated with resistance to fluoroquinolones, the real effect of the biological fitness of *S.* Typhi mutants has not been determined. Fitness can be determined in several ways, yet classically is usually determined by assessing the growth rate of bacteria in a suitable media [239]. Studies conducted with *S.* Typhimurium have shown that antimicrobial resistance conferred the fitness cost and caused the loss of ability to

colonize the gut of a chicken [203,220]. The biological cost of fluoroquinolone resistance has not been assessed in *S.* Typhi.

To investigate the impact that point mutations generate in fluoroquinolone resistant *S.* Typhi strains, we applied the allelic exchange methodology to reconstruct the common *gyrA* and *parC* mutations which were defined in Asian *S.* Typhi collection. To construct these mutants we used an attenuated *S.* Typhi strain, namely BRD 948. Here, to improve accuracy we developed and applied a pyrosequencing technique which is a quantitative method to measure the fitness cost of all reconstructed *S.* Typhi mutants. This pyrosequencing technique was carried out in the comparison with the classical colony counting method.

5.2 Results

5.2.1 Reconstruction of point mutations in *gyrA* and *parC* genes in *S.* Typhi

Eight point mutations were chosen for introduction into the *gyrA* and *parC* genes of the attenuated *S.* Typhi BRD 948 strain. These mutations generated five mutants which were detected in a collection of clinical isolates and two other unrelated natural mutations.

To make the individual mutants, DNA fragments which contained the target mutation were amplified by PCR before being digested and ligated into the suicide vector pJCB12. CC118λpir *E. coli* cells were transformed with the recombinant plasmid. The transformants which were able to grow on media supplemented with chloramphenicol were screened for transformation using specific *gyrA* (or *parC*) primers and plasmid primers (Figure 5.2). Colonies which gave positive PCR amplicons were subjected to plasmid extraction. Plasmid DNA was used for secondary transformation into *S.* Typhi

BRD 948 or a *S.* Typhi BRD 948 derived strain which already harbored mutation(s) in a different locus in order to achieve an additional substitution in the same or an additional gene.

S. Typhi recombinants were screened using specific primers for *gyrA* (or *parC*) incorporations. For instance, the primers 47125 and gyrA-11 (or parC-24) (Table 2.4) were used for screening *gyrA* (or *parC*) gene incorporation respectively. The integration of *gyrA* fragment into the DNA chromosome of the BRD 948 *S.* Typhi was achieved after exposure to chloramphenicol and ampicillin and selection on 5% sucrose. A high quality PCR product of the target DNA was prepared for sequencing analysis (Figure 5.3). A successful mutation reconstruction could only be determined if the nucleotide substitution was confirmed by comparing with the *S.* Typhi Ty2 DNA sequence, which was used as a reference (Figure 5.4).

Seven point mutations were engineered in the QRDR of the *gyrA* and *parC* genes of the attenuated BRD 948 *S.* Typhi strain (Table 5.1). Three of these mutants were single substitutions, namely, WT 144; Asp87→ Gly (or D87G, replacing GAC with GGC in the *gyrA* gene), WT 297; Asp87→Ala (or D87A, replacing GAC with GCC in the *gyrA* gene) gene and WT 304; Ser80→Ile (or S80I, replacing TCG with TAG in the *parC* gene).

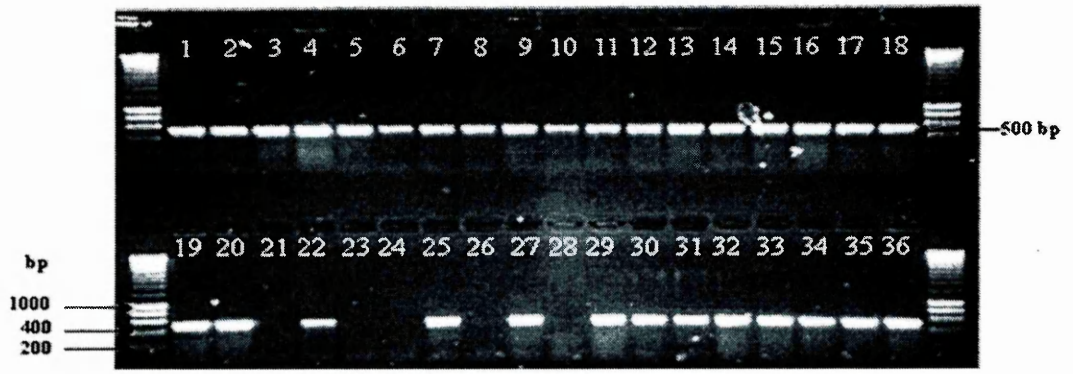


Figure 5.2 PCR screening for the incorporation of *gyrA* gene in *E. coli* transformants (500bp)

Colonies grown on selective media after electrotransformation were randomly selected for screening *gyrA* gene incorporation using primer pair R6K-01 and gyrA-53. Lane 1 → 20, 22, 25, 27, 29→36; positive bands represent the presences of transformed vector in CC118λpir *E. coli.* Land 21, 23, 24, 26 and 28; negative reactions, no plasmid transformed into CC118λpir *E. coli.* Hyper ladder I (Bioline, UK) was used as a base pair size marker.

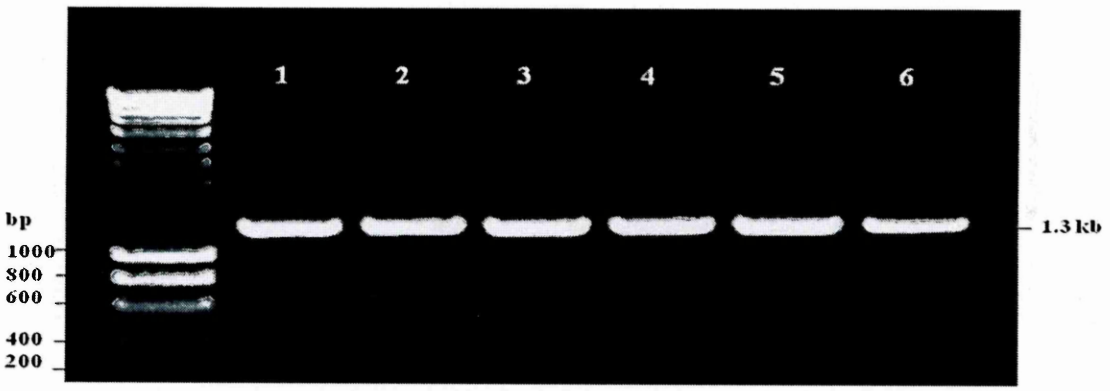


Figure 5.3 DNA amplification of the mutated *gyrA* gene of the BRD 948 *S.* Typhi derivatives

Pfu PCR was performed with colony 15.8.1 (NAL sensitive) (Lane 1), colony 15.8.2 (NAL resistance) (Lane 2), colony 15.8.5 (NAL resistance) (Lane 3), colony 25.14.1 (NAL sensitive) (Lane 4), colony 25.14.6 (NAL resistance) (Lane 5) and colony 25.14.11 (NAL resistance) (Lane 6). Hyper ladder I (Bioline, UK) was used. PCR fragment length was achieved using primers gyrA-31 and gyrA-11 (1.3kb).

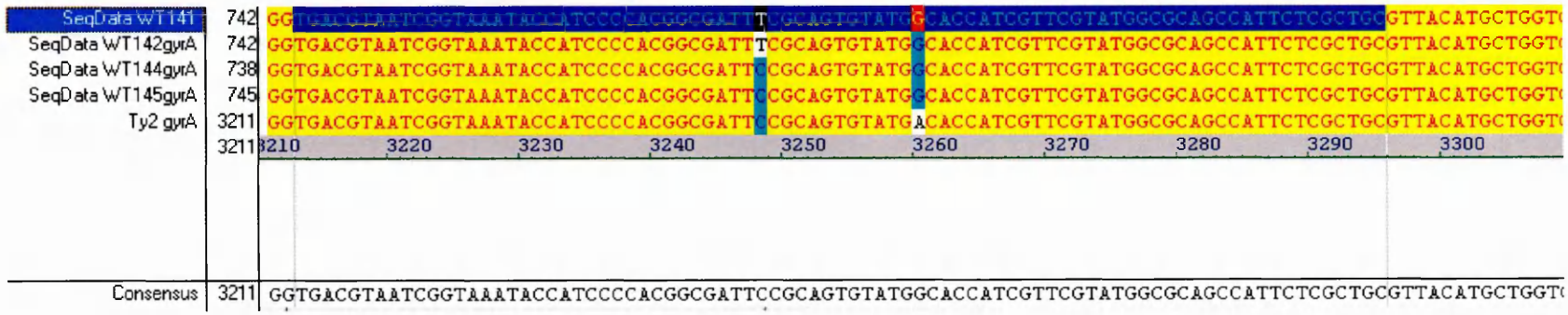


Figure 5.4 Representative nucleotide alignment with *S.* Typhi Ty2 with successful engineered *gyrA* mutants

The sequences of the *S.* Typhi derivatives, namely WT 142, WT 144 and WT 145 were aligned with *S.* Typhi Ty2 using Vector NTI suite 7.

Table 5.1 List of reconstructed mutations in *gyrA* and *parC* genes of the BRD 948 *S.* Typhi strain

The seven reconstructed mutations include the substitutions of Glycine (G) and Alanine (A) for Aspartic acid (D) at position 87, the substitutions of Phenylalanine (F) and Glycine (G) for Serine (S) at position 83 of the *gyrA* genes and the substitutions of Isoleucine (I) for Serine (S) at position 80 of the *parC* genes.

Mutation types (n=7)	*gyrA* gene		*parC* gene
Single (3)		D87G	
		D87A	
			S80I
Double (3)	S83F	D87G	
		D87G	S80I
	S83F	D87A	
Triple (1)	S83F	D87G	S80I

Three out of seven mutants included two substitutions. Amongst these three mutants, two of them were the double *gyrA* reconstructed mutants which were the WT 141; replacing Ser83 with Phe and Asp87 to Gly (or S83F and D87G), and the WT 300; replacing Ser83 with Phe and Asp87to Ala (or S83F and D87A) in the *gyrA* gene. One double mutant was constructed including a single *gyrA* mutation (Asp87→Gly) combined with a single *parC* mutation (Ser80→Ile), namely WT 287 (or D87G and S80I). The triple mutant; WT 286 was a combination of a double *gyrA* mutation, Ser83→Phe and Asp87→Gly and a single *parC* mutation Ser80→Ile (or S83FD87G and S80I). This triple mutant was constructed by introducing the Ser80Ile mutation in *parC* locus into the engineered WT 141 mutant (Ser83→Phe and Asp87→Gly). These seven newly constructed mutants were combined with the BRD 948 and four previously made mutants, namely WT 21; Ser83→Tyr (or S83Y, replacing TCC with TAC in the *gyrA* gene), WT 26; Ser83→Phe (or S83F, replacing TCC with TTC in the *gyrA* gene), WT 37; Asp87→Asn (or D87N, replacing GAC with AAC in the *gyrA* gene), WT 43;

Ser83→Phe and Asp87→Asn (or S83F & D87N), therefore assays were performed with a total of 12 strains, as shown in Table 5.2.

5.2.2 Fluoroquinolone susceptibilities of the *gyrA* and *parC* reconstructed *S.* Typhi mutants

To evaluate the susceptibilities to fluoroquinolones in the reconstructed *S.* Typhi mutants, we performed MIC testing on the reconstructed mutants using five fluoroquinolones and one quinolone, these were, nalidixic acid (NAL), ofloxacin (OFX), norfloxacin (NOX), ciprofloxacin (CIP), gatifloxacin (GAT) and levofloxacin (LVX) (Figure 5.5 and Table 5.2). The parental *S.* Typhi strain, BRD 948 and each reconstructed mutant were subjected to experimentation. The fluoroquinolone susceptibilities of 12 reconstructed mutants are represented in Table 5.2.

Table 5.2 The MIC of the *gyrA* and *parC* engineered *S.* Typhi mutants to fluoroquinolones

The MICs of nalidixic acid (NAL), norfloxacin (NOX), ofloxacin (OFX), ciprofloxacin (CIP), gatifloxacin (GAT) and levofloxacin (LVX) were performed to the *S.* Typhi BRD 948 strain and eleven *S.* Typhi reconstructed mutants. The *S.* Typhi reconstructed mutants were the single *gyrA* substitution at position 83 which include WT 21 (S83Y), WT 26 (S83F) and the single *gyrA* substitution at position 87 which include WT 297 (D87A), WT 37 (D87N), WT 144 (D87G); the double *gyrA* substitutions were WT 141 (S83F and D87G), WT 300 (S83F and D87A), WT 43 (S83F and D87N); a double mutant, WT 287, which was the combination of a single *gyrA* (D87G) mutation and a single *parC* mutation (S80I); a triple mutants, WT 286, which was the combination of a double *gyrA* (S83F and D87G) mutations and a single *parC* mutation (S80I); and a single *parC* mutant, WT 304 (S80I).
(*) Kindly provided by the Sanger Institute, UK.

No	ID	Origin	Description	MIC (µg / mL)					
				NAL	NOX	OFX	CIP	GAT	LVX
1	BRD 948*	parental	No mutation	1.5	0.064	0.047	0.008	0.008	0.012
2	WT21*	engineered	S83Y	>256	0.5	0.25	0.125	0.125	0.125
3	WT26*	engineered	S83F	>256	0.75	0.38	0.125	0.125	0.125
4	WT297	engineered	D87A	48	0.75	0.19	0.094	0.064	0.064
5	WT37*	engineered	D87N	48	0.75	0.25	0.125	0.125	0.125
6	WT144	engineered	D87G	48	0.75	0.25	0.125	0.125	0.25
7	WT287	engineered	D87G and S80I(*parC*)	>256	1	0.25	0.125	0.094	0.094
8	WT141	engineered	S83F and D87G	>256	1	0.38	0.19	0.25	0.25
9	WT300	engineered	S83F and D87A	192	1.5	0.38	0.25	0.38	0.25
10	WT43*	engineered	S83F and D87N	64	0.75	0.38	0.19	0.19	0.19
11	WT286	engineered	S83F and D87G and S80I (*parC*)	>256	24	16	8	2	3
12	WT304	engineered	S80I(*parC*)	3	0.19	0.047	0.016	0.016	0.016

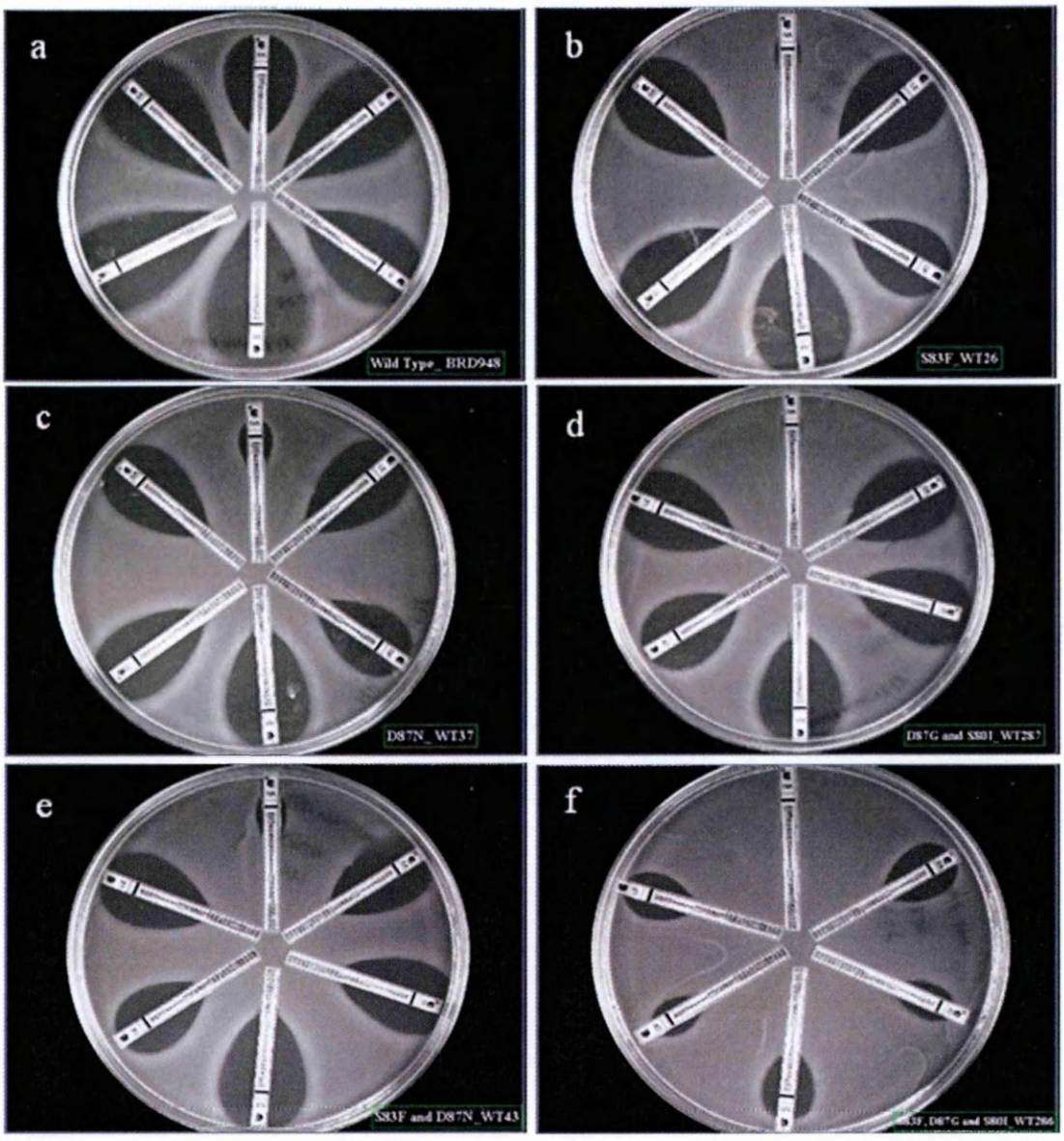


Figure 5.5 MIC testing of the engineered *S.* Typhi strains performed by E-test

Six different patterns of fluoroquinolone MIC to reconstructed *S.* Typhi mutants are depicted. a; the wild type BRD 948 strain, b and c; single mutant WT26 (S83F) and WT37 (D87N) respectively, d; double mutant WT 287 (D87G in *gyrA* and S80I in *parC* genes), e; the double mutant WT43 (S83F and D87N), and f; the triple mutant WT286 (S83F, D87G and S80I). The reconstructed mutants were exposed to six (fluoro)quinolones, in which nalidixic acid was embedded at the 12 o'clock direction and the other antimicrobials are in clockwise order including gatifloxacin, ciprofloxacin, norfloxacin, ofloxacin and levofloxacin. The MIC values were interpreted following CLSI guideline and are represented in Table 5.2.

5.2.2.1 The impact of the single and double substitution(s) in the *gyrA* gene on the MICs to nalidixic acid

To understand the impact of the different reconstructed mutations on the MICs of (fluoro)quinolones, we compared the MICs of nalidixic acid of the differing mutations to the parental *S.* Typhi strain, BRD 948. Almost all of the reconstructed *gyrA* and *parC* mutants showed resistance to nalidixic acid (Table 5.2). The MICs to nalidixic acid of the reconstructed mutants ranged from 48 μg / mL with WT 37 (D87N), 64 μg / mL with WT 297 (D87A) and WT 43 (S83F and D87N) to higher than 256 μg / mL with single substitutions in WT 26 (S83F), WT 21 (S83Y) and WT 144 (D87G). The MIC to nalidixic acid was also higher than 256 μg / mL with the double mutants WT 141 (S83F and D87G), WT 300 (S83F and D87A), WT 287 (D87G and S80I (*parC*)) and the triple mutant WT 286 (S83F and D87G and S80I (*parC*)) (Table 5.2).

Although MICs to nalidixic acid with the mutants was dependant on the location of the site of the mutation, the nalidixic acid resistant mutants could be grouped into 3 subgroups. The low nalidixic acid resistance group included WT 37 (D87N), WT 144 (D87G) and WT 297 (D87A) (NAL MIC 48 mg / mL); the intermediate resistance group, which included two double mutants, WT 43 (S83F and D87N) and WT 300 (S83F and D87A) (NAL MIC 64 μg / mL and 192 μg / mL, respectively); and the high level resistance group, which included the single substitutions, WT 21 (S83Y), WT 26 (S83F); the double substitutions, WT 287 (D87G and S80I(*parC*)), WT 141 (S83F and D87G); and the triple mutation WT 286, (S83F, D87G and S80I (*parC*)) with NAL MIC >256 μg / mL. The single *parC* mutant, WT 304, was the only mutant which was susceptible to nalidixic acid with NAL MIC 3 μg / mL.

The amino acid substitutions including Phenylalanine or Tyrosine for Serine at position 83 of the *gyrA* gene depicted more resistance to nalidixic acid than the substitutions (Glycine, Alanine and Asparagine) for Aspartic acid at position 87 of the *gyrA* gene. The combination of S83F and D87N produced the lowest MIC to nalidixic acid (NAL MIC 64 μg / mL) compared with other double mutations and the single mutation S83F (NAL MIC >256 μg / mL). The double mutation, WT 287 (D87G (*gyrA*) and S08I (*parC*)) showed increased MIC of nalidixic acid compared with the single mutation D87G with NAL MIC >256 μg / mL and 48 μg / mL, respectively (Table 5.2).

5.2.2.2 The impacts of the single and double substitution(s) in *gyrA* gene on the MIC to fluoroquinolones

All the single and double reconstructed *S.* Typhi mutants were susceptible to the fluoroquinolones, according to the current standard CLSI guidelines (norfloxacin, ofloxacin, ciprofloxacin, gatifloxacin and levofloxacin). The fluoroquinolone MICs of the single and double reconstructed *gyrA* mutations did not significantly vary. These MICs ranged from 0.5 μg / mL to 1.5 μg / mL for norfloxacin; from 0.19 μg / mL to 0.38 μg / mL for ofloxacin, from 0.094 μg / mL to 0.25 μg / mL for ciprofloxacin, from 0.094 μg / mL to 0.38 μg / mL for gatifloxacin and from 0.094 μg / mL to 0.25 μg / mL for levofloxacin (Table 5.2). In comparison with the parental BRD 948, the MIC mean to ofloxacin with the single and double *gyrA* mutants was 6-fold higher. The MIC means to norfloxacin, ciprofloxacin, gatifloxacin and levofloxacin were 12, 17, 19 and 12-fold higher than the MIC value of the BRD 948 strain, respectively (Table 5.3). Thus, the single and double reconstructed *gyrA* mutations had the lowest increase in the MIC to ofloxacin when compared with that of other fluoroquinolones.

Table 5.3 The correlation of (fluoro)quinolone MIC with various *S.* Typhi reconstructed mutants and *S.* Typhi BRD 948

The increasing fold of MICs of nalidixic acid (NAL), norfloxacin (NOX), ofloxacin (OFX), ciprofloxacin (CIP), gatifloxacin (GAT) and levofloxacin (LVX) were performed by dividing the MIC of BRD 948 by MIC of mutant.

No	*S.* Typhi reconstructed mutant group	MIC increase (folds)					
		NAL	NOX	OFX	CIP	GAT	LVX
1	Single , double *gyrA* mutants	na	12	6	17	19	12
2	Double *gyrA* and single *parC* mutant*	na	375	340	1000	250	250
3	Single *parC* mutant	2	3	1	2	2	1

(*) the triple mutant

5.2.2.3 The impact of the triple mutation double *gyrA* mutations (S83FD87G) and a single *parC* mutation (S80I) on the MIC to fluoroquinolones

The MICs of the triple mutant to fluoroquinolones, demonstrated a significant increase when compared with those of the *S.* Typhi BRD 948 strain. The triple mutant stimulated a 250-fold increase in the MICs to gatifloxacin and levofloxacin compared with the MICs of these antimicrobials to the parental BRD 948 (GAT MIC 0.008 μg / mL and LVX 0.012 μg / mL). The triple mutant demonstrated a 340-fold and 375-fold increase in MIC to ofloxacin and norfloxacin, respectively, when compared to the parent *S.* Typhi strain (OFX MIC 0.047 μg / mL and NOX MIC 0.064 μg / mL). The highest MIC increase to this triple mutant was stimulated by ciprofloxacin (1000-fold increase) compared with the MIC of ciprofloxacin to the BRD 948 strain (MIC 0.008 μg / mL).

5.2.2.4 The impact of the single *parC* reconstructed mutant on the MICs of fluoroquinolones

A single *parC* mutation has never been reported in clinical isolates. In this study, this mutant was engineered in order to reveal its own role on the fluoroquinolone resistance. The MICs of fluoroquinolones to the single *parC* reconstructed mutant were equal to or

a 1-fold increase than MICs of those antimicrobials to BRD 948 *S.* Typhi strain and the highest MIC increase (3-fold) compared with BRD 948 of this mutant was the MIC of norfloxacin (Table 5.3).

5.2.2.5 The bactericidal activity of fluoroquinolones

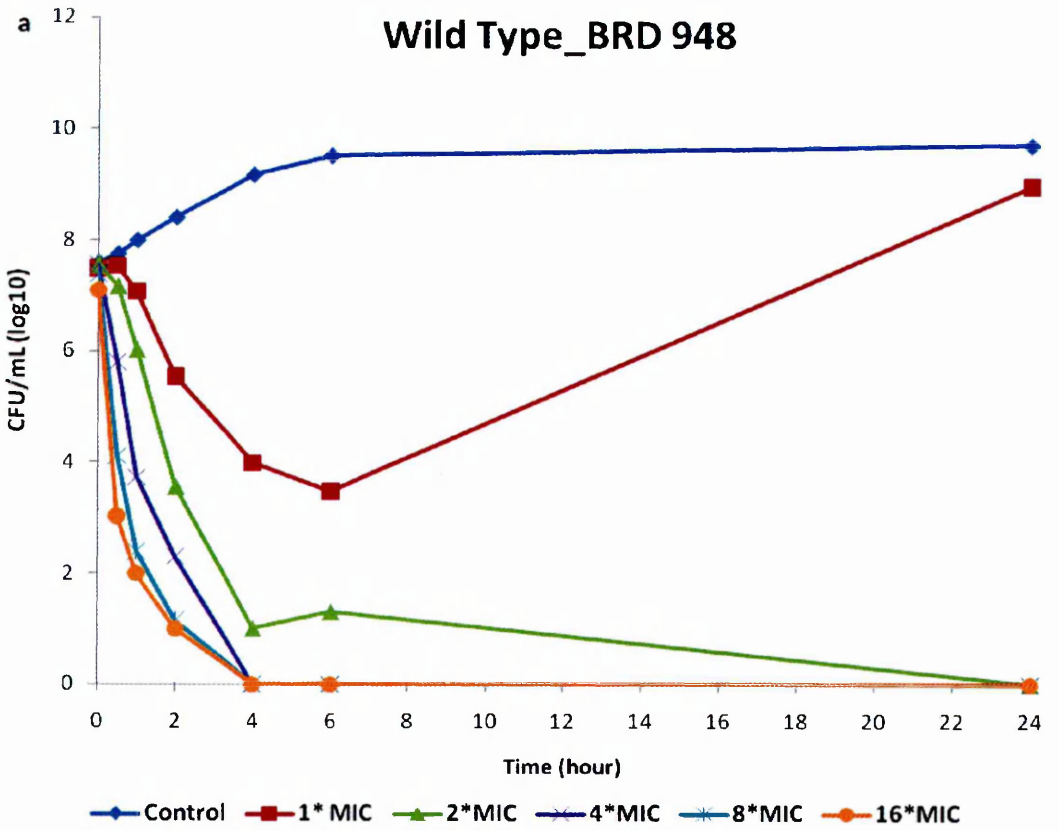
It is noticeable that some mutants showed similar resistance levels to some fluoroquinolones. The similar fluoroquinolone resistance pattern was found among norfloxacin, ofloxacin and ciprofloxacin and the lowest MIC to highest MIC of these antimicrobials were in the following order D87A / S83Y / D87N / D87G / D87G and S80I / S83F and D87N / S83F / S83F and D87G / S83F and D87A / S83F, D87G and S80I (*parC*). Whilst a similar fluoroquinolone MIC pattern was seen with gatifloxacin MICs and levofloxacin MICs, the strains with the lowest to the highest MIC with these antimicrobials followed this order; D87A / S83Y / D87N / D87G / D87G and S80I / S83F and D87N / S83F / S83F and D87G / S83F and D87A / S83F, D87G and S80I (*parC*). Interestingly, the double mutant WT 287 (D87G and S80I (*parC*)) showed a linear MIC decrease following the order of fluoroquinolone generation from old to new (nalidixic acid / norfloxacin / ofloxacin, ciprofloxacin / gatifloxacin and levofloxacin).

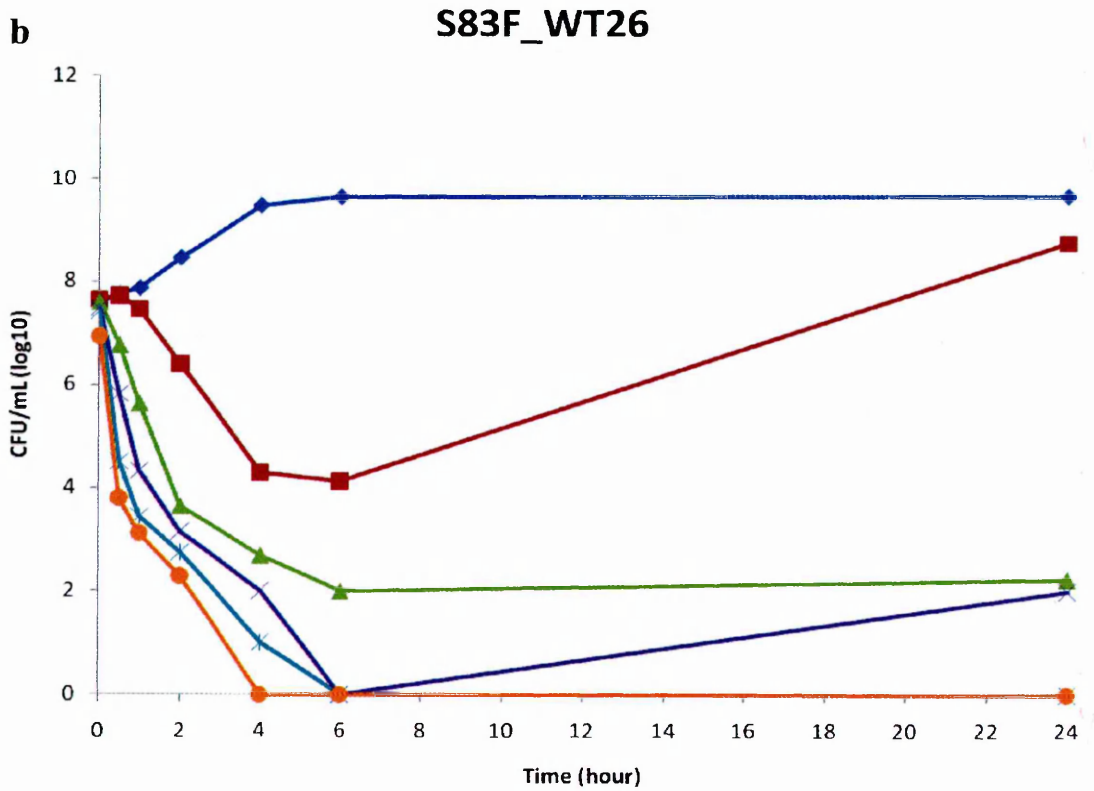
Although the MICs of (fluoro)quinolones were not statistically significantly related to the amino acid substitutions in the target enzymes of the *S.* Typhi strains, the mutation combinations provided evidence of the mutation roles to the level of fluoroquinolone resistance in *S.* Typhi. The level of fluoroquinolone resistance was dependent on the antimicrobial generation, i.e. the ofloxacin MIC level of single and double *gyrA* mutant group compared to that of the BRD 948 was a 6-fold increase, but the gatifloxacin MIC level was a 19-fold increase. The fluoroquinolone resistance mutation combinations

giving the highest MIC was the substitution S83F combined with either the D87G or the D87A, and the triple mutants S83F, D87G and S80I (*parC*).

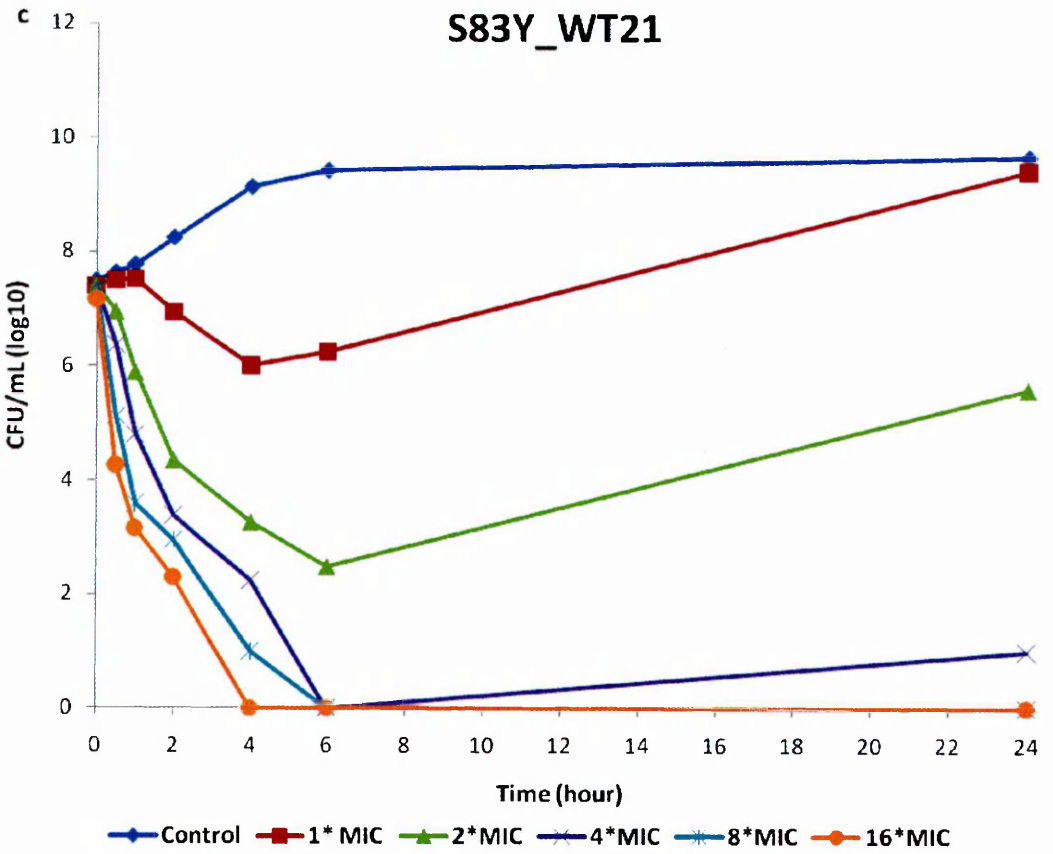
5.2.3 Time – kill analysis

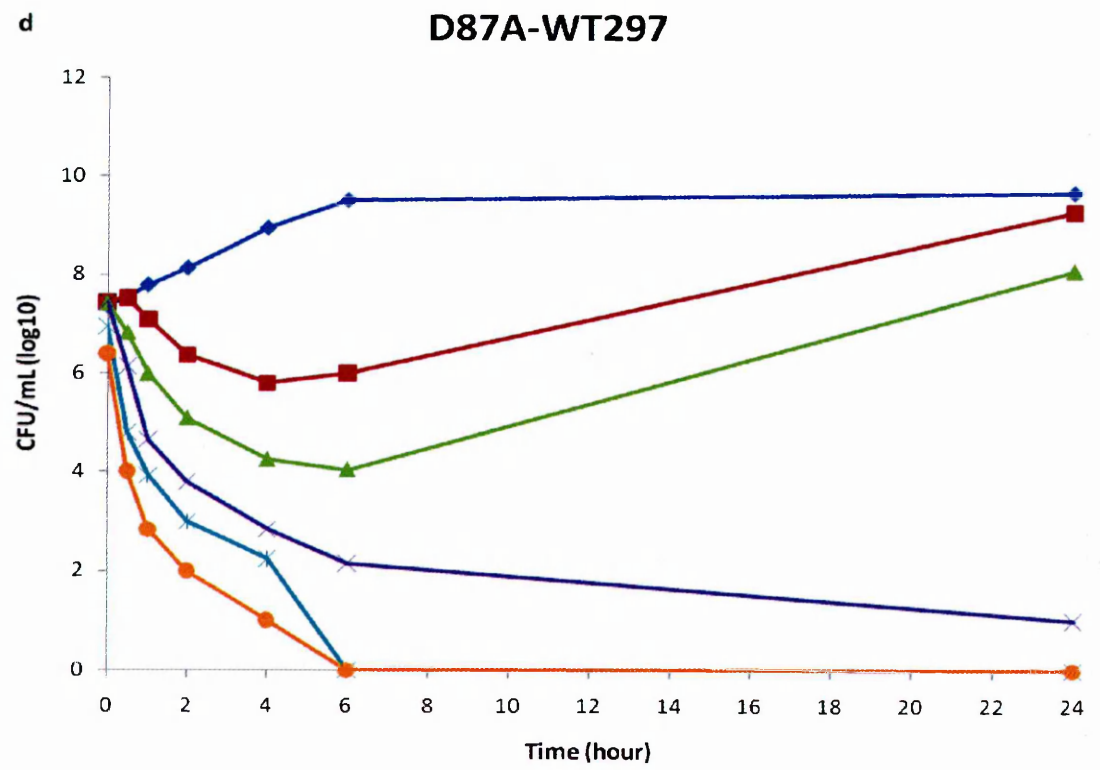
The susceptibilities of the *S.* Typhi reconstructed mutants to fluoroquinolones were demonstrated by the MICs investigation. However, the MICs only depicted the minimum concentration of antimicrobials to inhibit bacterial growth; the effective bactericidal dose of fluoroquinolones to each mutant required further investigation. To investigate the responses of each amino acid substitution reconstructed in the *S.* Typhi strains to the bactericidal of ofloxacin, each of the reconstructed mutant was challenged with different concentrations of ofloxacin (1x, 2x, 4x, 8x and 16x MIC). The rate of survival was evaluated at six time points as before (30 minutes, 1 hour, 2 hours, 4 hours, 6 hours and 24 hours) (Figure 5.5).



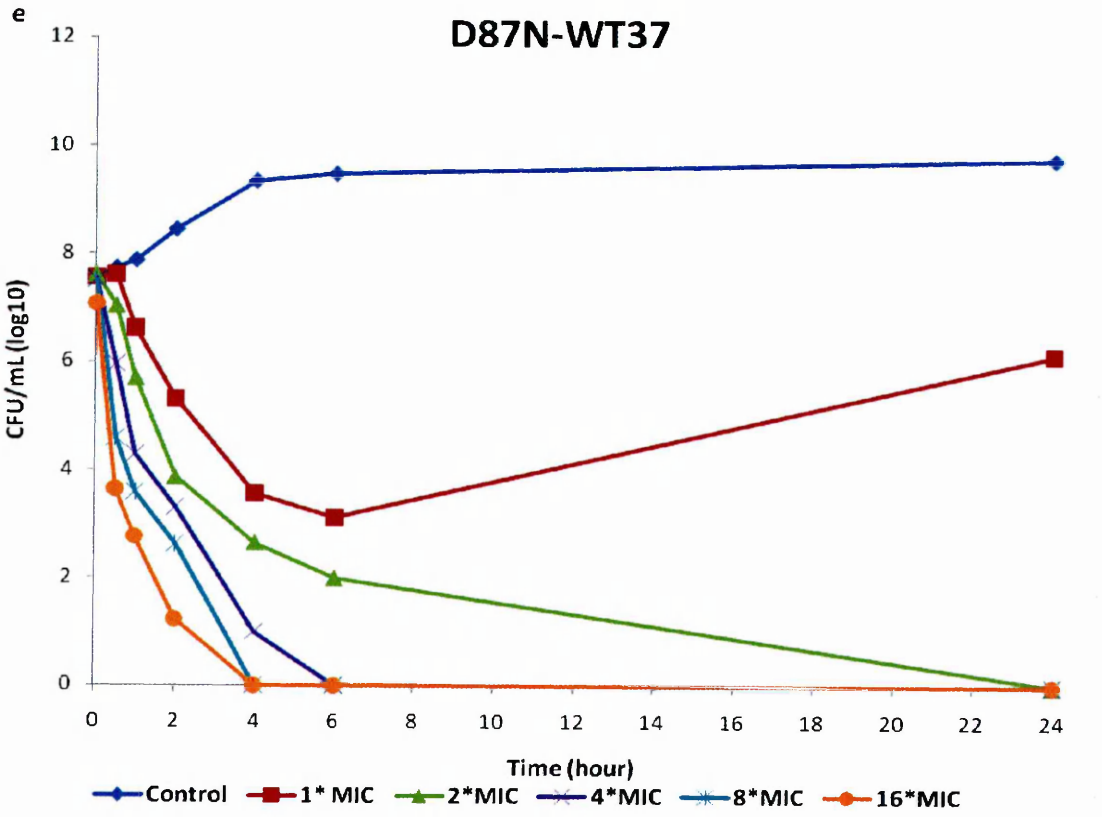


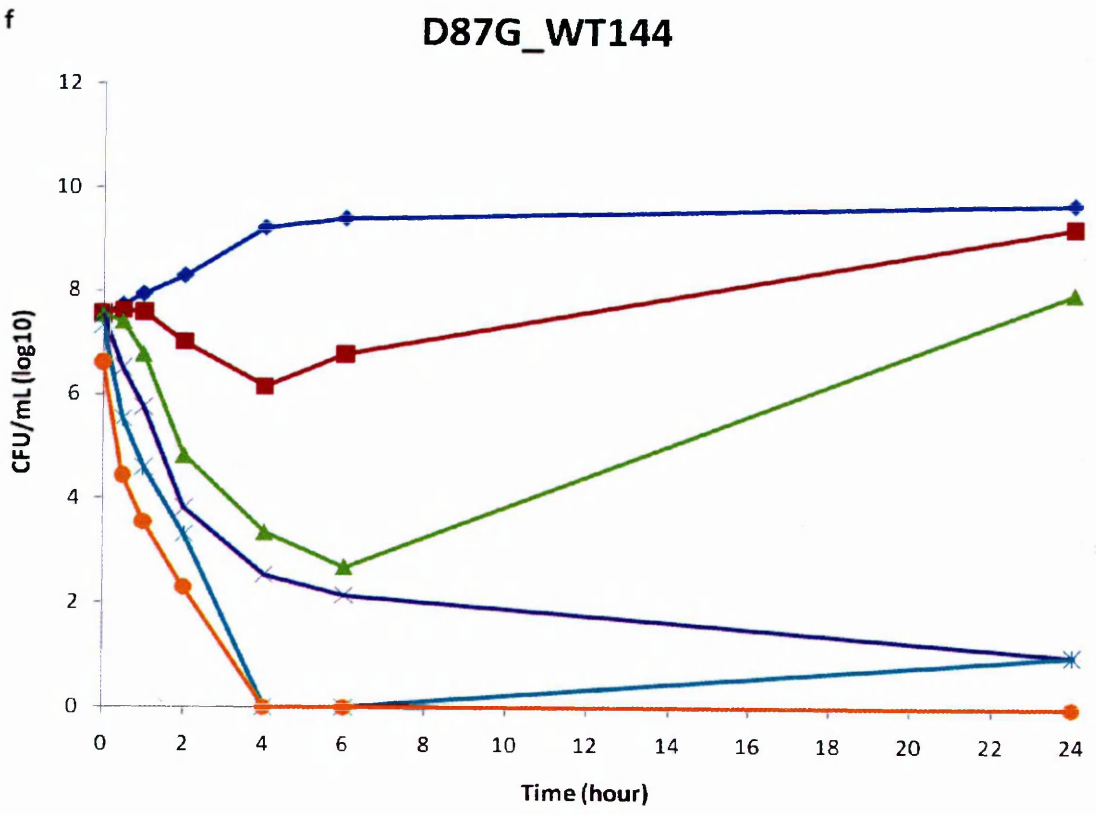
Wild type strain BRD 948 (a), *S.* Typhi WT 26 (Ser83→Phe) (b) were exposed to ofloxacin.



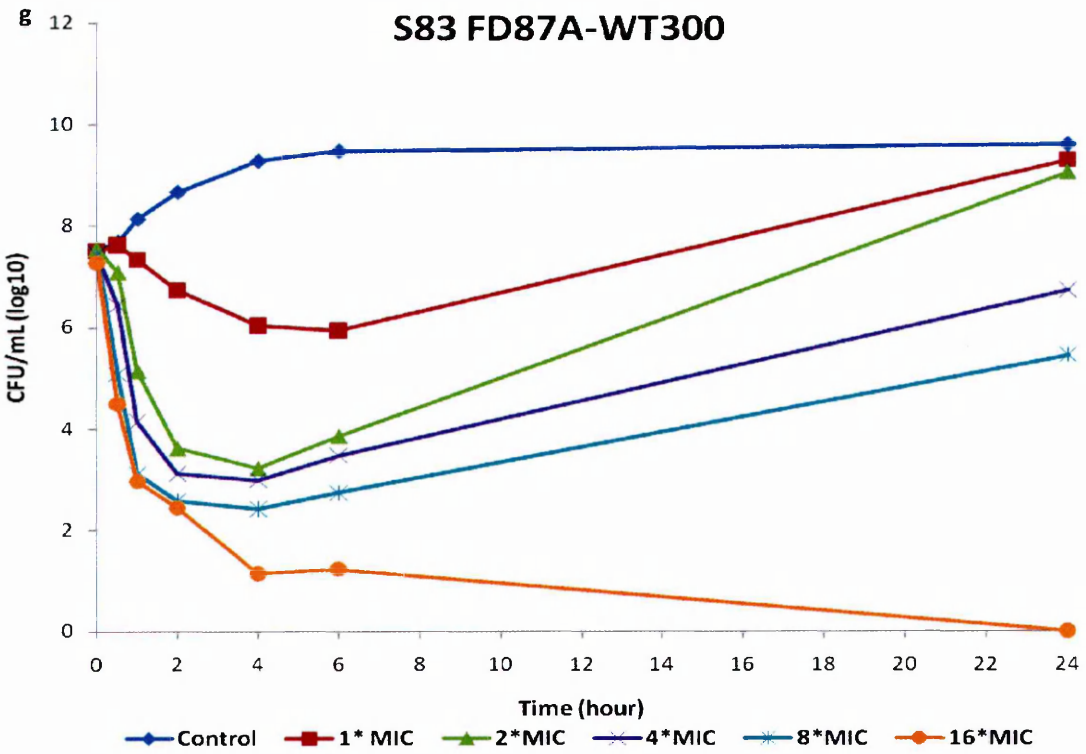


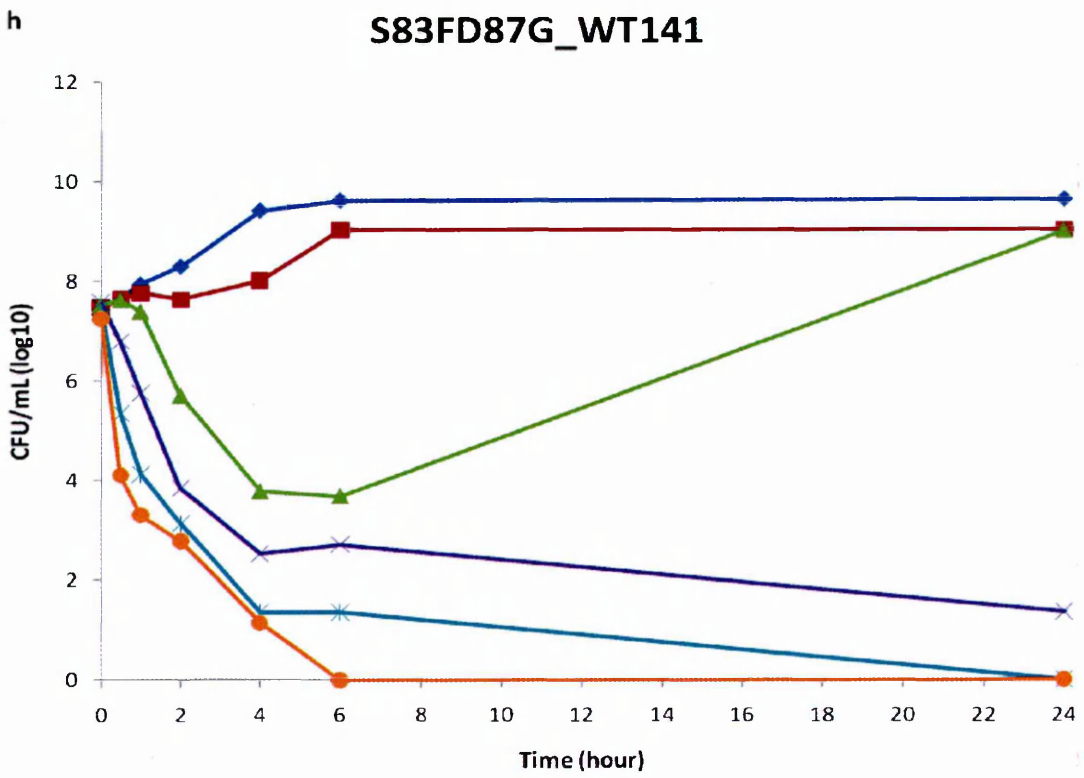
S. Typhi WT 21 (Ser83→Tyr) (c) and WT 297 (Asp87→Ala) (d) were exposed to ofloxacin.



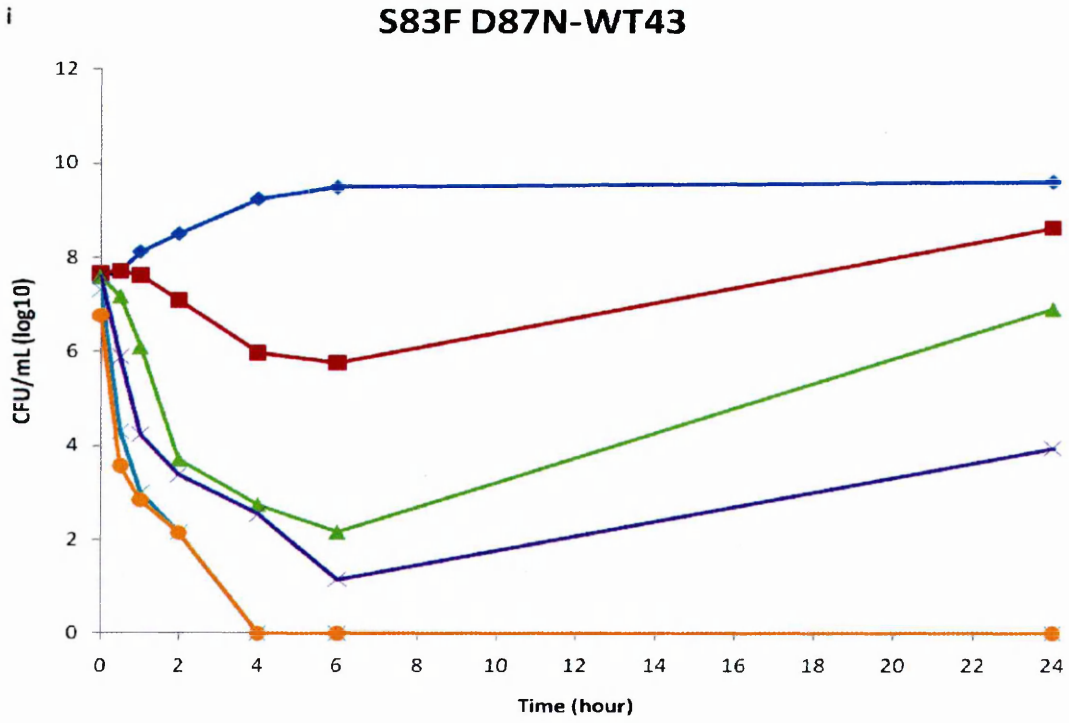


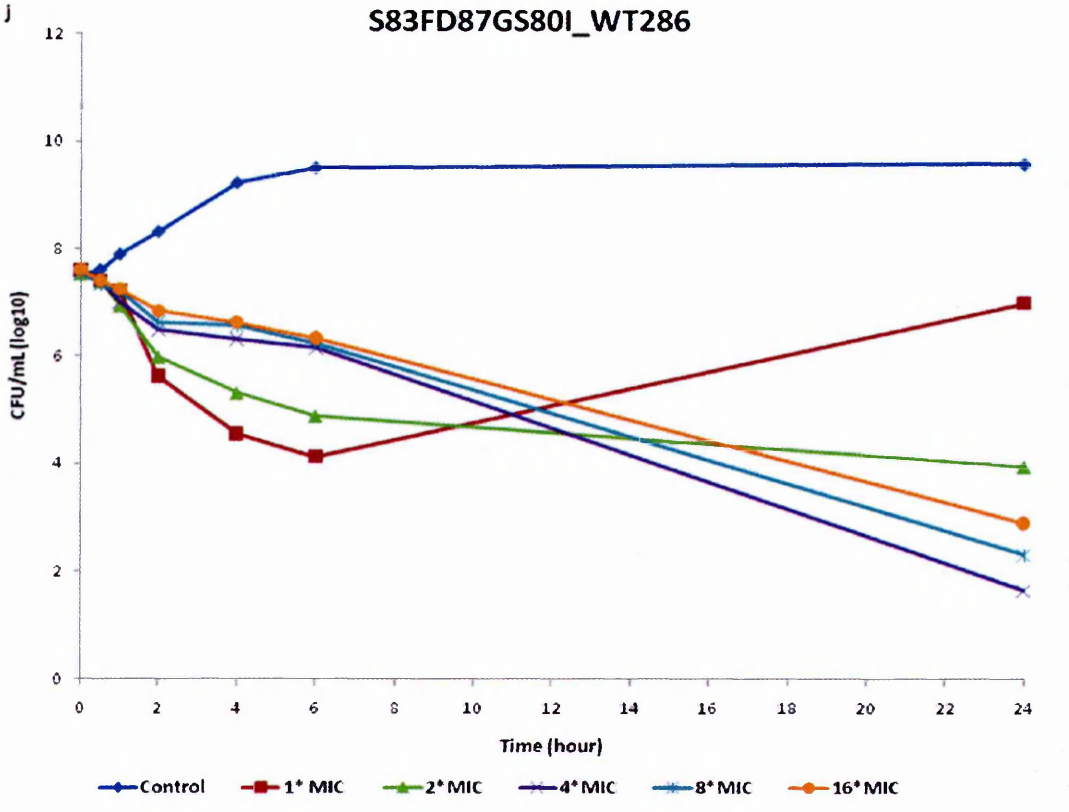
S. Typhi WT 37 (Asp87→Asn) (e) and *S.* Typhi WT 144 (Asp87→Gly) (f) were exposed to ofloxacin.



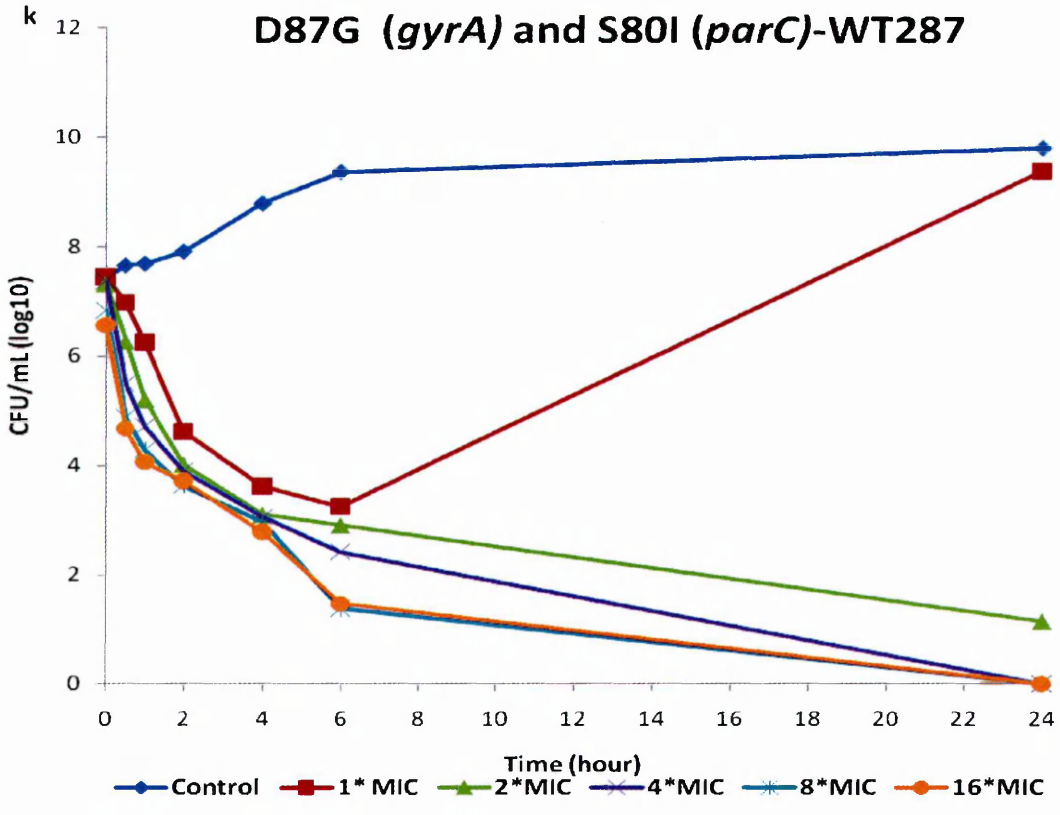


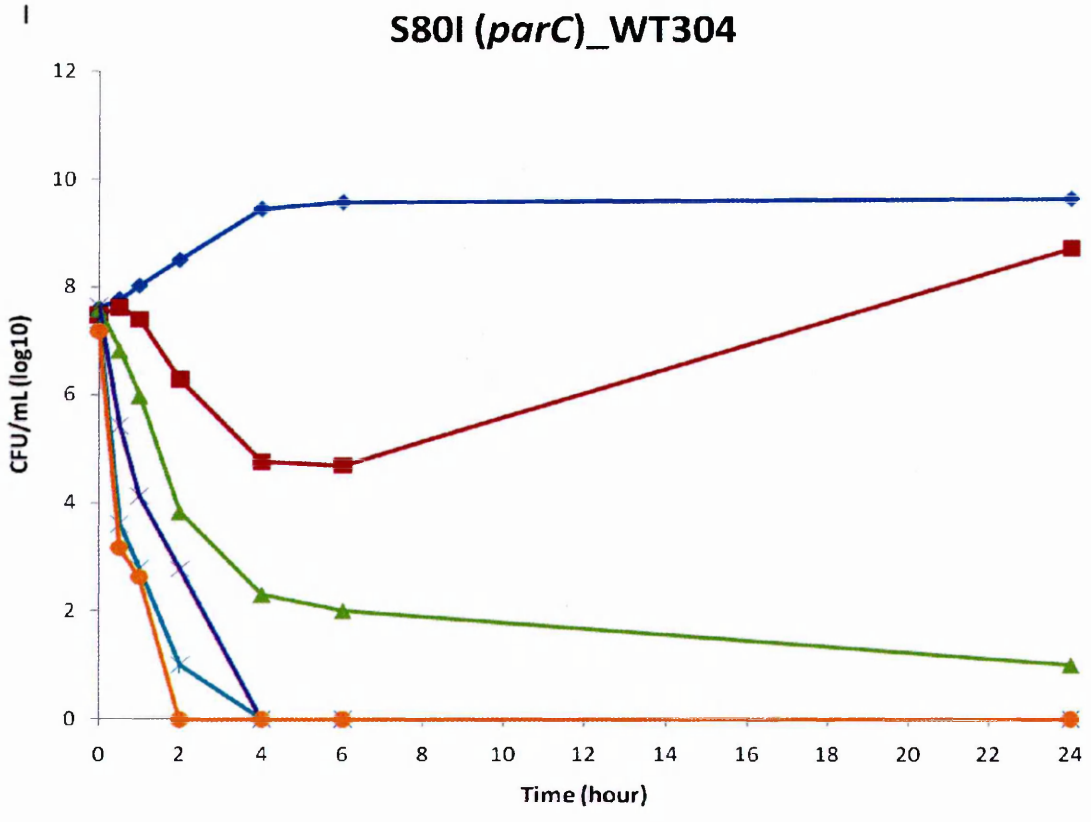
S. Typhi WT 300 (Ser83→Phe and Asp87→Ala) (g) and *S.* Typhi WT 141 (Ser83→Phe and Asp87→Gly) (h) were exposed to ofloxacin.





S. Typhi WT 43 Ser83→Phe and Asp87→Asn) (i) and *S.* Typhi WT 286 (Ser83→Phe and Asp87→Gly in *gyrA* and Ser80→Ile in *parC*) (j) were exposed to ofloxacin.





S. Typhi WT 287 (Asp87→Gly in *gyrA* and Ser80→Ile in *parC*) (k) and *S.* Typhi WT 304 (Ser80→Ile in *parC*) (l) were exposed to ofloxacin.

Figure 5.5 *In vitro* time - kill experiments of reconstructed *gyrA* and *parC* mutations in *S.* Typhi

In vitro experiments were performed in duplicate and results represent the mean of duplicate values, errors with standard deviation. The y axis represents the $\log_{10}$ value of *S.* Typhi CFU / mL. The x axis represents the time line and the *S.* Typhi growth was sampled at six time points, 0, 30 minutes, 1, 2, 4, 6 and 24 hours (section 2.6). Each line depicts an individual of ofloxacin concentration and the MIC concentrations used against the organism tested. The subtitle of each diagram represents mutation type and name of the mutant.

Each mutation group was selected for *in vitro* time - kill experiments. The mean changes in $\log_{10}$ CFU / mL over the 24 hour period are presented in Figure 5. 5.

Ofloxacin was bactericidal with respect to *S.* Typhi BRD 948, at all concentrations (2x, 4x, 8x and 16x MIC) except 1x MIC and gave total bacterial killing after 4 hours. A similar killing rate of ofloxacin to the WT 304, the single *parC* mutation (S80I), was also observed. In general, ofloxacin was fully bactericidal to all the mutants at 8x MIC and 16x MIC of ofloxacin after 24 hours. However, the killing rates of ofloxacin on differing mutants varied. No bactericidal effect was shown against the triple mutant (WT 286) with ofloxacin.

5.2.3.1 The bactericidal effect of ofloxacin to the single mutants

Bactericidal activity of ofloxacin to the single substituted mutants was also seen after 4 hours, although the killing activity was not as high as when compared to the killing of the wild type strain. Four times the MIC of ofloxacin caused a decrease of bacterial concentrations of WT 26 (S83F), WT 21 (S83Y), WT 144 (D87G), WT 37 (D87N) and WT 297 (D87A) mutants from 10^7 CFU / mL to between 10^2 and 10^3 CFU / mL. Eight times and 16x MIC of ofloxacin concentrations killed all the single engineered mutants.

5.2.3.2 The bactericidal activity of ofloxacin to the double mutants in comparison with the single and triple mutants

The mean *S.*Typhi inoculum calculated from all mutants against 4 times MIC of ofloxacin at the start of the killing assay (Time 0) was 3.3×10^7 CFU/mL (2.95×10^7 - 3.65×10^7, CI 95%). Therefore, the results calculated after 4 hours exposure to 4 times MIC of ofloxacin were dependant on the experimental behavoiur of the individual mutants and not the initial starting innoculum.

An overall bactericidal activity of 4x MIC of ofloxacin was not observed with all the double substituted mutants, this concentration only caused a decrease in the bacterial concentration from 10^7 CFU / mL to 10^3 CFU / mL. A small difference in the killing efficacy of ofloxacin was observed with WT 287, (D87G (*gyrA*) and S80I (*parC*)), as this strain showed more resistance to ofloxacin than other double reconstructed *S.* Typhi mutants. Eight times and 16x MIC of ofloxacin caused a reduced bacterial concentration from 10^7 CFU / mL to 10^3 CFU / mL. In addition, WT 287 (D87G (*gyrA*) and S80I (*parC*)) was also more resistant to ofloxacin than WT 144 (D87G) which has the same substitution at position 87 of the *gyrA* gene but harbored no substitution in *parC.* The triple mutant, WT 286 (S83F, D87G and S80I), was completely resistant to all MIC levels of ofloxacin.

Regardless of the highest ofloxacin MIC in the triple mutant, there was no correlation between the number of mutation and ofloxacin resistance level in *S.* Typhi reconstructed strains (*p* linear trend was 0.003, *p* value for linear regression is significant when it is less than 0.001) (Figure 5.6).

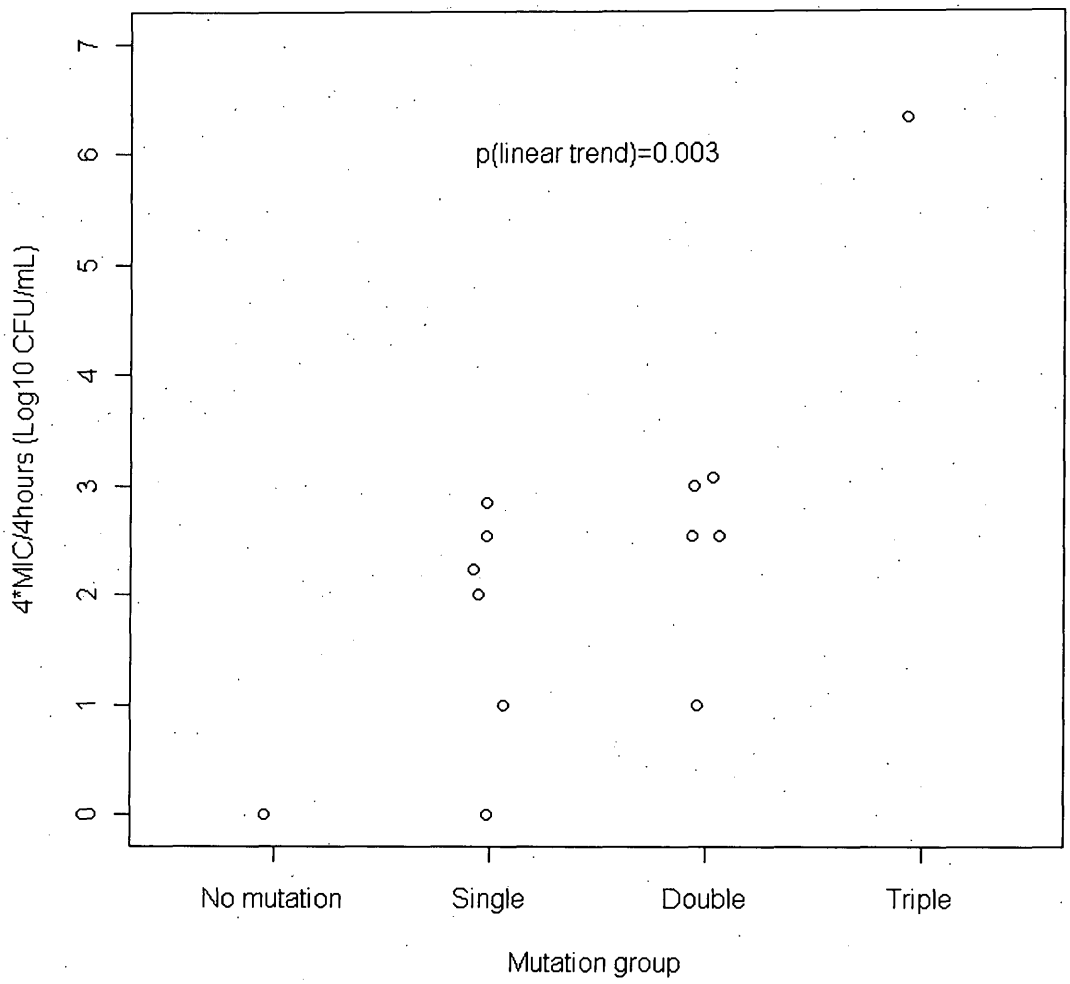


Figure 5.6 The killing rate relationship of ofloxacin to different reconstructed *S.* Typhi mutants and the *S.* Typhi BRD 948 strain

The y axis represents the $\log_{10}$ CFU of the reconstructed *S.* Typhi strains after 4 hours of exposure to 4x MIC of ofloxacin. The x axis represents 4 *S.* Typhi groups including non - mutant (BRD 948), single, double and triple mutation groups. Each point represents mean of $\log_{10}$CFU / mL of an individual *S.* Typhi strain (n = 13). *p* linear trend was 0.003 using linear regression test.

5.2.4 Biological cost of reconstructed *gyrA* and *parC S.* Typhi mutants

The point mutations in *gyrA* and *parC* genes of the *S.* Typhi strains caused nalidixic resistance and reduced fluoroquinolone susceptibilities. These *S.* Typhi strains have also been reported to be persistent and transmissible globally [147,162,190,191,199,226,232-234]. Moreover, it has been shown that their transmission and dissemination are not only affected by the environment and host factors, but also are influenced by the relative fitness of the drug resistant organisms in the absence of selective pressure [240]. We assessed the fitness cost of the reconstructed *S.* Typhi mutants both in growth supportive media (LB aro) and under stress in a high concentration of oxgall. We also calculated the selective cost of each of the reconstructed *S.* Typhi mutants in competition with the parental BRD 948 strain.

5.2.4.1 Independent growth

The growth rates of the reconstructed mutants in LB aro were measured separately. The representatives of eight reconstructed mutants were selected for this growth assay. These strains included the parent *S.* Typhi strain and the reconstructed *S.* Typhi mutants which were WT 144 (D87G), WT 37 (D87N), WT 26 (S83F), WT 300 (S83F and D87A), WT 141 (S83F and D87G), WT 286 (S83F, D87G and S80I) and WT 304 (S83I). The growth was performed in LB supplemented with 1 % aro mix and the absorbance measurements were taken every 10 minutes over 24 hours (section 2.5.1). The mean of 2 independent growth cycles of each reconstructed *S.* Typhi strain is represented in Figure 5.7.

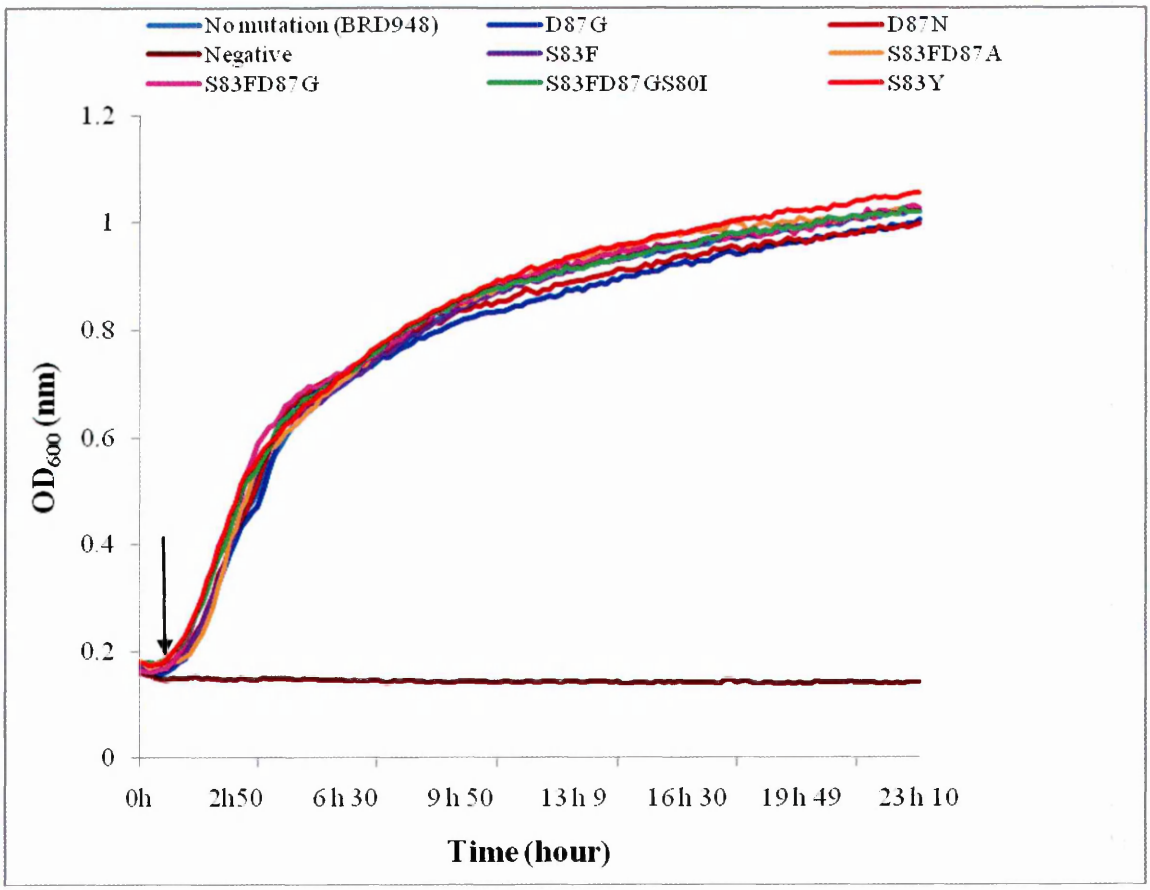


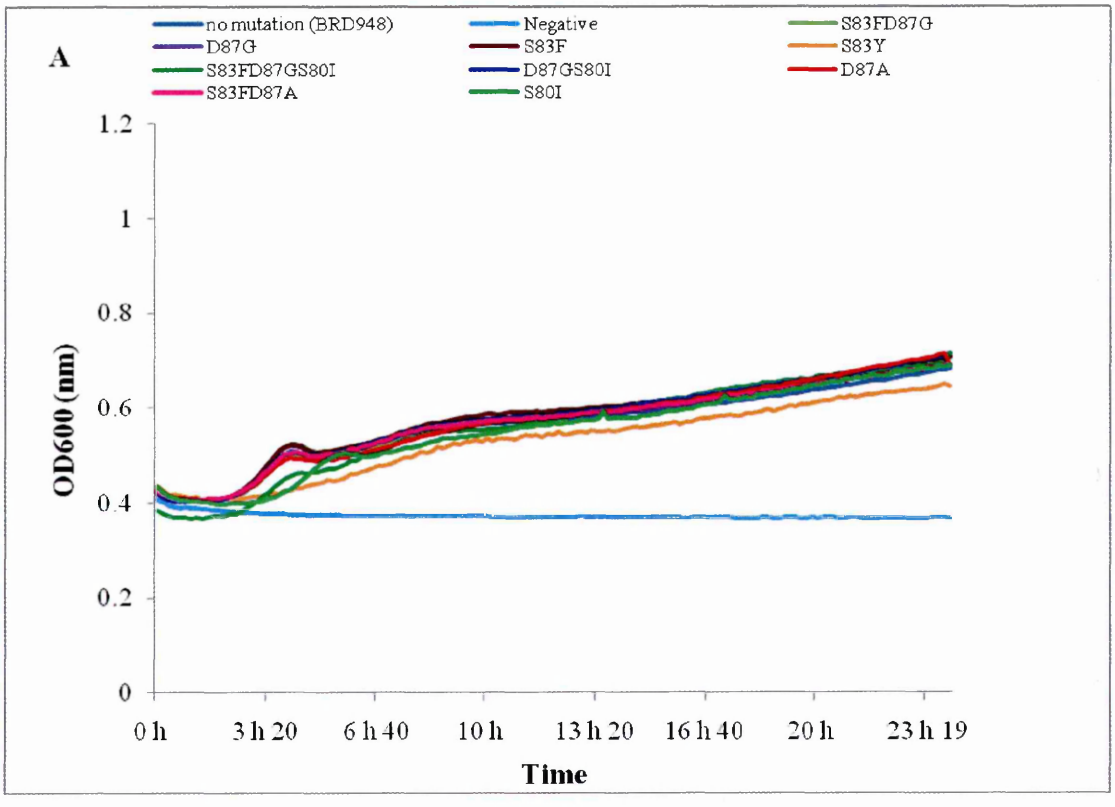
Figure 5.7 The growth of the reconstructed *S.* Typhi mutants in LB aro

Absorbance value is displayed as OD_{600} (y axis). Growth experiments were conducted in duplicate. The *S.* Typhi strains used in this experiment were the parental *S.* Typhi BRD 948 strain and the reconstructed *S.* Typhi mutants including WT 144 (D87G), WT 37(D87N), WT 21 (S80Y), WT 26 (S80F), WT 300 (S83F and D87A), WT 141 (S83F and D87G) and the triple mutant WT 286 (S83F, D87G (*gyrA*) and S80I (*parC*)). Negative was LB aro in which no bacteria were inoculated. The arrow represents the end of lag phase at time point 40 minutes.

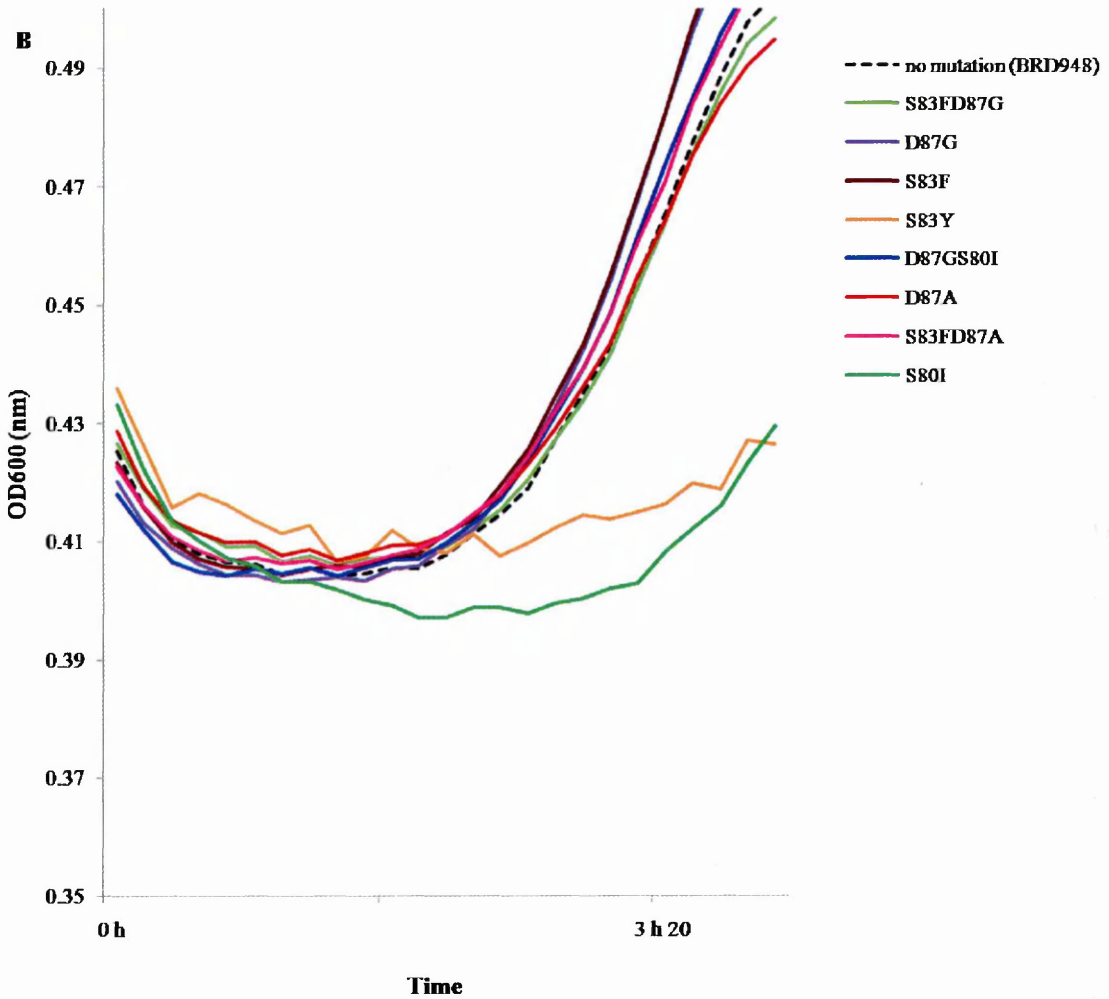
All of the reconstructed *S.* Typhi mutants observed had similar growth rate to the parental *S.* Typhi BRD 948 strain, there was no variation in the growth over a 24 hour period with these strains.

The reconstructed *S.* Typhi mutants were also challenged in LB aro medium supplemented with 8 % oxgall (Section 2.3). Eight percent oxgall was chosen to evaluate the growth abilities in stress media of reconstructed mutants since the growth of these mutants showed the most diversity in this concentration compared with their

growth in LB aro supplemented with different oxgall concentrations (0.0193 % - 7 %) (Section 2.3). Meanwhile, the growth of the reconstructed mutants in LB aro supplemented with higher concentrations of oxgall (9 % - 12 %) was not reproducible (Section 2.3). In 8 % oxgall media, we performed the growth experiment for 10 reconstructed *S.* Typhi mutants along with BRD 948 *S.* Typhi strain used as a control. Figure 5.8 shows the growth dynamics of all reconstructed *S.* Typhi strains in 8 % oxgall over 24 hours. The mean of each mutant growth rate was calculated from 9 replicates in three separate experiments.



(A) The growth of the reconstructed *S.* Typhi mutants in oxgal 8 % in 24 hour.



(B) The growth of the reconstructed *S.* Typhi mutants in oxgal 8 % after 4 hours.

Figure 5.8 The growth of the reconstructed mutants in LB aro supplemented with 8 % oxgall

Absorbance value is displayed as OD_{600}. The growth experiments were conducted in LB aro containing 8 % oxgal and the means were calculated from 9 replicates in three separate experiments. Eleven *S.* Typhi strains for this experiment include the parental *S.* Typhi BRD 948 (no mutation) and the reconstructed *S.* Typhi mutants including WT 144 (D87G), WT 297 (D87A), WT 21 (S80Y), WT 26 (S80F), WT 300 (S83F and D87A), WT 141 (S83F and D87G), WT 287 (D87GS80I), WT 304 (S80I) and the triple mutant WT 286 (S83FD87G (*gyrA*) and S80I (*parC*)). The number represents the end of lag phase at time point 1 hour 50 minutes.

All of the *S.* Typhi strains showed reduced growth rates in LB aro 8 % oxgall compared to their growth in LB aro (Figure 5.8A and Figure 5.7). The lag phase of growth of the reconstructed mutants in 8 % oxgall was extended by 1 hour and 50 minutes in comparison with their growth in LB aro medium without oxgall. In fact, the lag growth phase of the reconstructed mutants in LB aro was 40 minutes. Additionally, the growth of BRD 948 *S.* Typhi and the reconstructed mutants in LB aro 8 % oxgall did not reach similar stationary phase with those grown in LB aro without oxgall. In addition, the growth rates in LB aro with 8 % oxgall after 4 hours depicted that the single mutants WT 21 (S83Y) and the WT 304 (S80I (*parC*)) had reduced the growth rates compared with other *gyrA* mutants including WT 144 (D87G), WT 297 (D87A), WT 21 (S80Y), WT 26 (S80F), WT 300 (S83F and D87A), WT 141 (S83F and D87G) and WT 287 (D87G and S80I) (Figure 5.8B). This would indicate that these strains would reach the exponential phase slower, even though they had the same growth kinetics with other mutants after 24 hours. Both WT 21 (S83Y) and WT 304 (S80I (*parC*)) showed the same OD_{600} means 0.43 nm at 4 hours compared with OD_{600} means 0.50 nm of the BRD 948 strain at the same time point.

5.2.4.2 Competition growth

Only subtle differences between strains were noticeable in the 24 hour growth period monitored by independent growth assays. To assess any potential growth advantage or disadvantage between the strains over a prolonged period we performed protracted mixed culture competition assays as previously performed to assess bacterial fitness in *E. coli* [241]. The reconstructed mutants were subjected to the competition assay against the parental *S.* Typhi BRD 948 strain.

Two methods were applied for this competition investigation. Initially, the classical colony counting was applied to determine the viable bacteria of each *S.* Typhi strain. However, owing to the large dilution factors and potential fluctuations in growth rate we found that colony counting only gave a marker of either negative or positive selective growth effects. The method of subtracting the number of organisms grown of selective media from those grown on non - selective media was not found to be reproducible over the 15 day subculture period. Data showing an inherent error in the assay is presented in Figure 5.9; fluctuations were seen in the ratio of organisms over the 15 days and with all the mutants. A linear regression test was performed on all the assays (Table 5.4) and was found to be none significant over the fifteen day growth period. Linear regression models the relationship between one or more variables denoted y and one or more variable denoted x, in this case proportion of bacteria at each time point. In the case of the colony counting method, none of the assays were deemed to be statistically significantly higher or lower than 0. However, whilst a pattern of increase or decrease in mutant proportions could be observed over fifteen days, this was highly variable and non - reproducible (Figure 5.9).

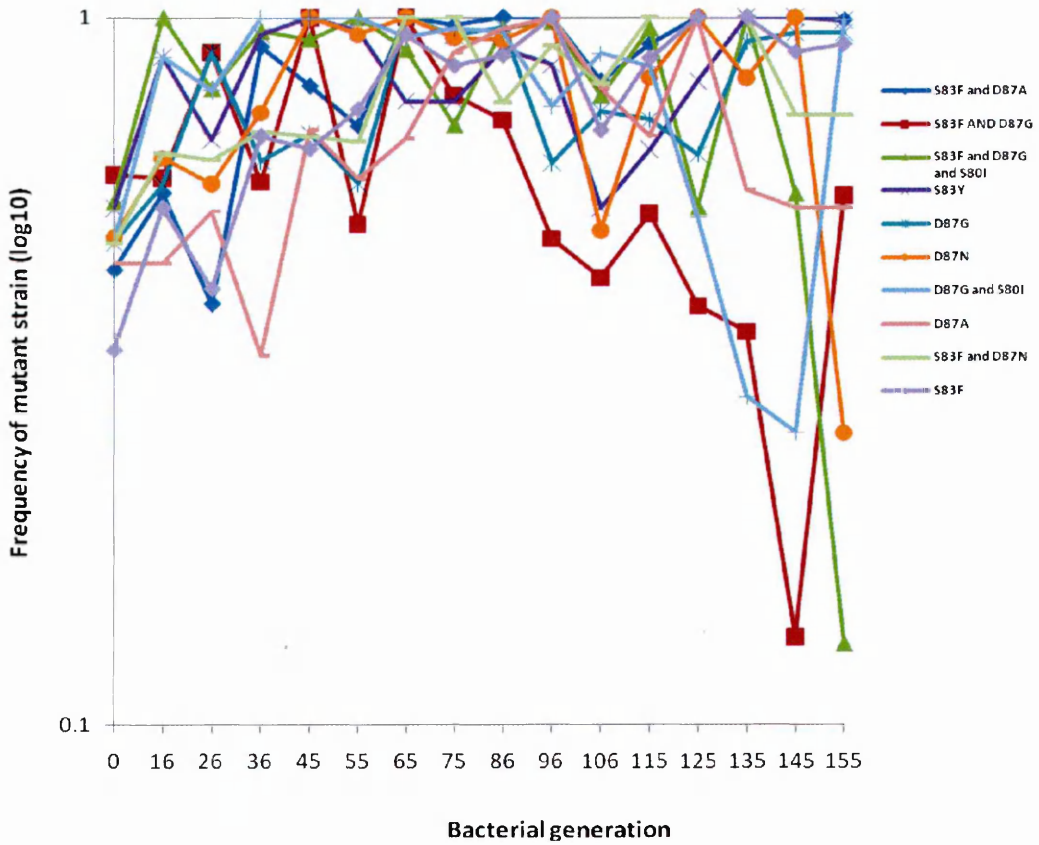


Figure 5.9 Plots of colony counting from competitive growth assays

All strains were mixed in a 1:1 ratio and sub-cultured daily for 15 days. Bacterial generations were assessed by calculating the number of doubling events from the initial inoculation and the final stationary phase concentration of bacteria. The frequency of resistance was calculated by subtracting the number of organisms grown on selective media from those grown on non - selective media. Each coloured line represents the frequency a different strain with respect to the parent strain over time, dark blue; WT 300, red; WT141, dark green; WT 286, purple; WT 21, light green; WT 144, orange; WT 37, light blue; WT 287, pink; WT 297, turquoise; WT 43 and lilac, WT 26.

Colony counting at each time point was performed not only to establish the ratio of mutant to wild type but also to calculate the numbers of inoculated bacteria per day and, therefore, we could accurately calculate bacterial doubling time and generations. The number of bacterial generations (which is dependent on inoculum size) was highly reproducible over 3 independent growth assays and between all mutants with the non - mutant. In total we calculated that each individual assay went through 9 - 11 generations per 24 hour period (after a prolonged stationary phase). Over the fifteen day period the organisms went through between 134 and 158 generations, this was not significantly variable between differing assays or strains (data not shown). Therefore, these values were used for all experimental assays.

To improve sensitivity, accuracy and reproducibility we developed and ultimately applied a pyrosequencing method to calculate bacterial proportions in the assay. The competition assays were performed as with colony counting, yet population changes were assessed using a pyrosequencing method specific for the SNP loci in the *gyrA* gene (Figure 5.11). The pyrosequencing method calculates the ratio of a SNP in a single position in a mixed reaction after PCR amplification (Figure 5.11). The pyrosequencing was compared with colony counting and known dilutions of mixed organisms were compared in order to validate the assay procedure. Figure 5.10 shows a comparison of the colony counting method and the pyrosequencing method with strain *S.* Typhi WT 21 and *S.* Typhi BRD 948. Whilst the overall change over 155 generations is same, the pyrosequencing method can be seen to be more accurate and the linear regression coefficient of the pyrosequencing method is statistically significantly greater than 0. This demonstrates a selective advantage of *S.* Typhi WT 21 over the parental strain *S.* Typhi BRD 948, which can be detected by pyrosequencing but not by colony counting.

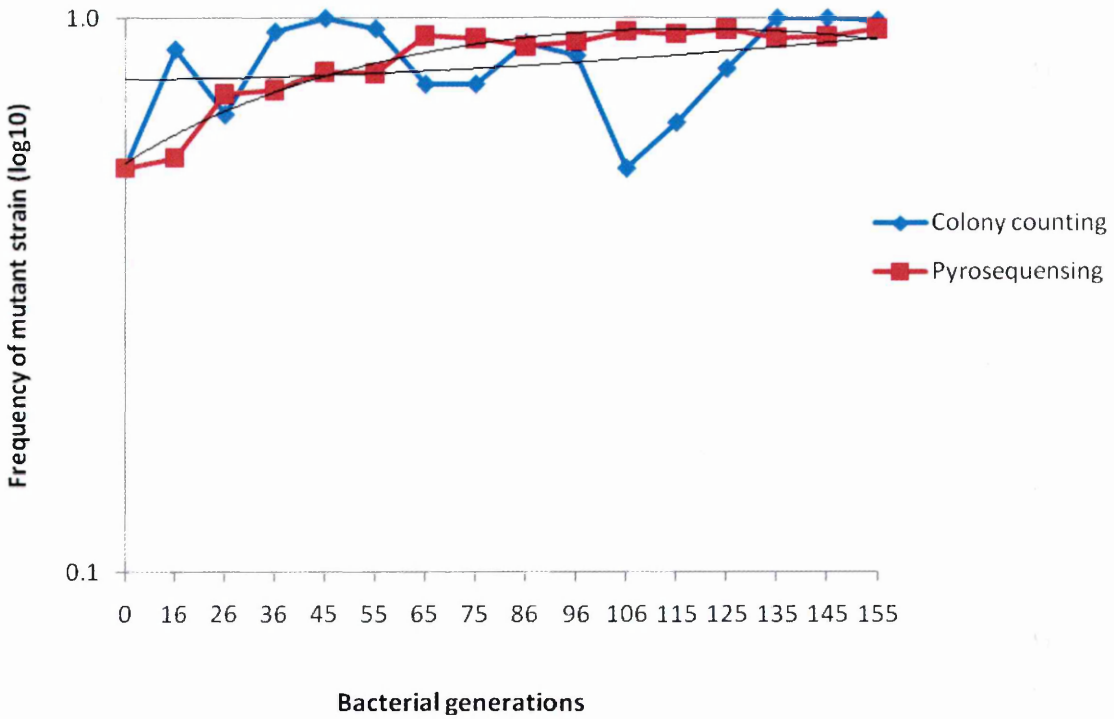


Figure 5.10 Comparison of colony counting and pyrosequencing for measuring competitive growth between two strains

The proportion of mutant to non - mutant was calculated by two different methods from the same assay for the strain *S.* Typhi WT 21 (S83Y) competed with *S.* Typhi BRD 948. This assay was performed over 15 days (155 generations). The general trend is an increase in the proportion of the mutant strain with both of the techniques, colony counting; blue and pyrosequencing; red. This tendency is shown with a polynomial trend line, colour corresponds as above. The linear regression for the colony counting (-0.067178) with r^2=0.0891 is none significant; however a significant linear regression is shown with the pyrosequencing method (0.1277) with r^2=0.9397.

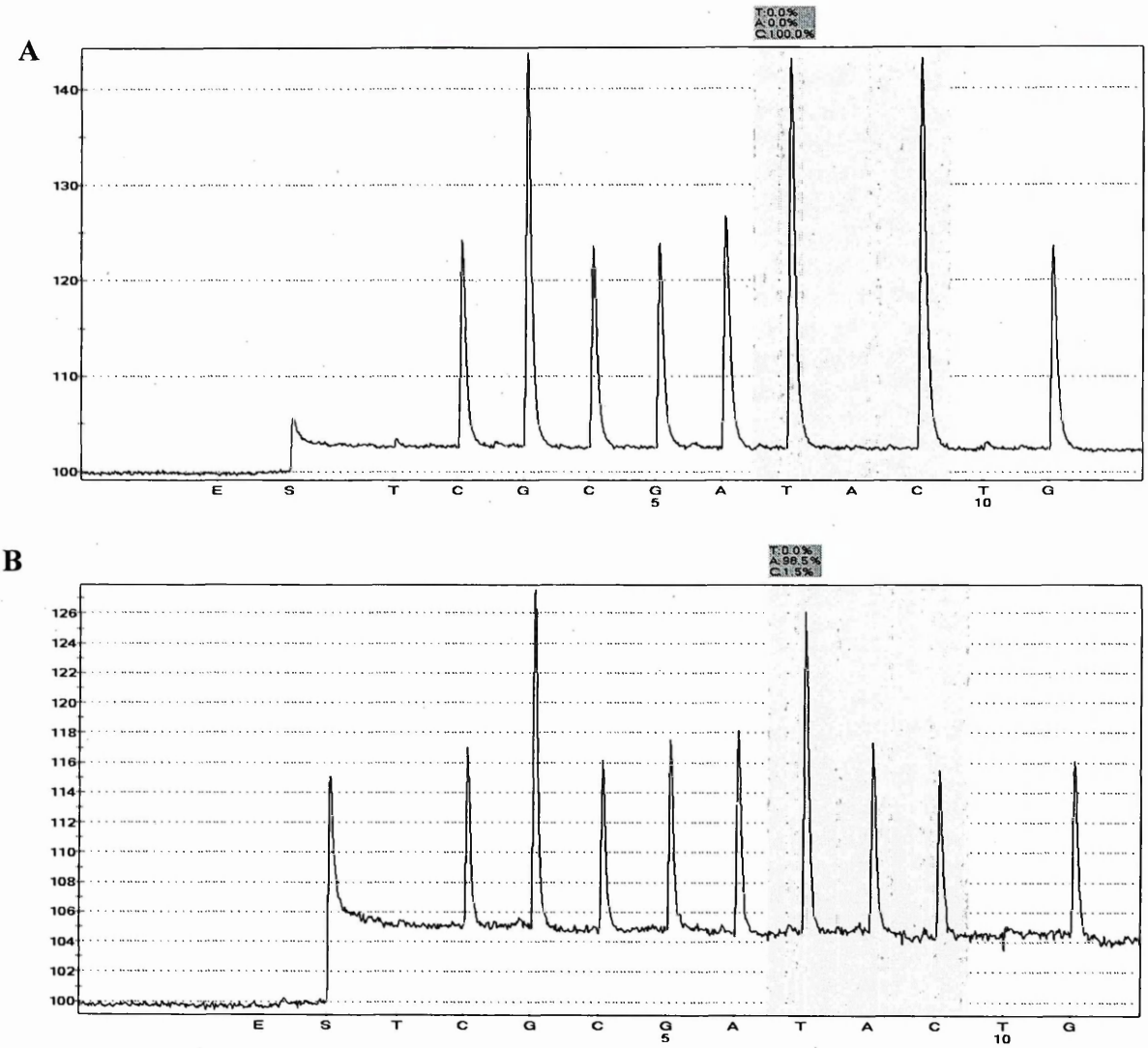


Figure 5.11 An example of py-program pyrosequencing traces detecting the ratio of a SNP between a reconstructed *S.* Typhi mutant in competition with *S.* Typhi BRD 948

(A) The py-program depicts 100% Cytosine (C) at locus 83 of the *gyrA* gene in the non - mutant *S.* Typhi BRD 948 strain. In comparison (B) detects 98.5% of Adenosine (A) in the same location of the *gyrA* gene in a mixed assay of *S.* Typhi BRD 948 and WT 21 (S83Y) which has a substitution of TAC for TCC at position 83 of the *gyrA* gene.

We performed competitive assays of all 10 mutants with the parental *S.* Typhi BRD 948 strain and detected the ratio of mutant to non - mutant with the pyrosequencing method. The data for these assays are presented in Table 5.4 and Figure 5.12. Table 5.4 shows the change in percentage and the corresponding linear regression, selection co-effiecient and relative fitness over the 15 days of the assay which included 155 bacterial generations. The selection coefficient (S) and relative fitness (F) were calculated by the linear regression model using a method previously described by Lindgren *et al.* [242]. The *in vitro* selection coefficient (S) per generation of each mutant can be calculated by linear regression as the slope [(number of mutant cells / number of wild-type cells) / (number of generations / number of cycles)] x ln2. Relative fitness (F) (with respect to *S.* Typhi BRD 948) is defined as 1 + S. Additionally, to ensure reproducibility the assays were performed in triplicate, and the ratio of mutant to non - mutant was calculated on day 0 and at the final time point on day 15 (Table 5.4).

Table 5.4 Calculation of relative fitness of ten reconstructed *S.* Typhi mutants

Assay	Mutant	Day 0	Day 15	Change in % / 15 days	Changein % / day	Linear Regression	Selection coefficient	Relative fitness
BRD948/WT300	S83F and D87A	0.490	0.020	-0.470	-0.031	-0.0058*	-0.004020254	0.995979746
BRD948/WT141	S83F and D87G	0.410	0.840	0.430	0.029	0.0437*	0.030290532	1.030290532
BRD948/WT286	S83F, D87G and S80I	0.460	0.130	-0.330	-0.022	-0.006*	-0.004158883	0.995841117
BRD948/WT21	S83Y	0.540	0.957	0.417	0.028	0.1277*	0.088514895	1.088514895
BRD948/WT144	D87G	0.510	0.620	0.110	0.007	0.0079*	0.005475863	1.005475863
BRD948/WT37	D87N	0.550	0.350	-0.200	-0.013	0.0003	0.000207944	1.000207944
BRD948/WT287	D87G and S80I	0.530	0.930	0.400	0.027	0.0997*	0.069106774	1.069106774
BRD948/WT297	D87A	0.550	0.940	0.390	0.026	0.0826*	0.057253957	1.057253957
BRD948/WT43	S83F and D87N	0.530	0.940	0.410	0.027	0.0687*	0.047619211	1.047619211
BRD948/WT26	S83F	0.440	0.660	0.220	0.015	0.0051*	0.003535051	1.003535051

* Significantly greater or less than zero with linear regression model

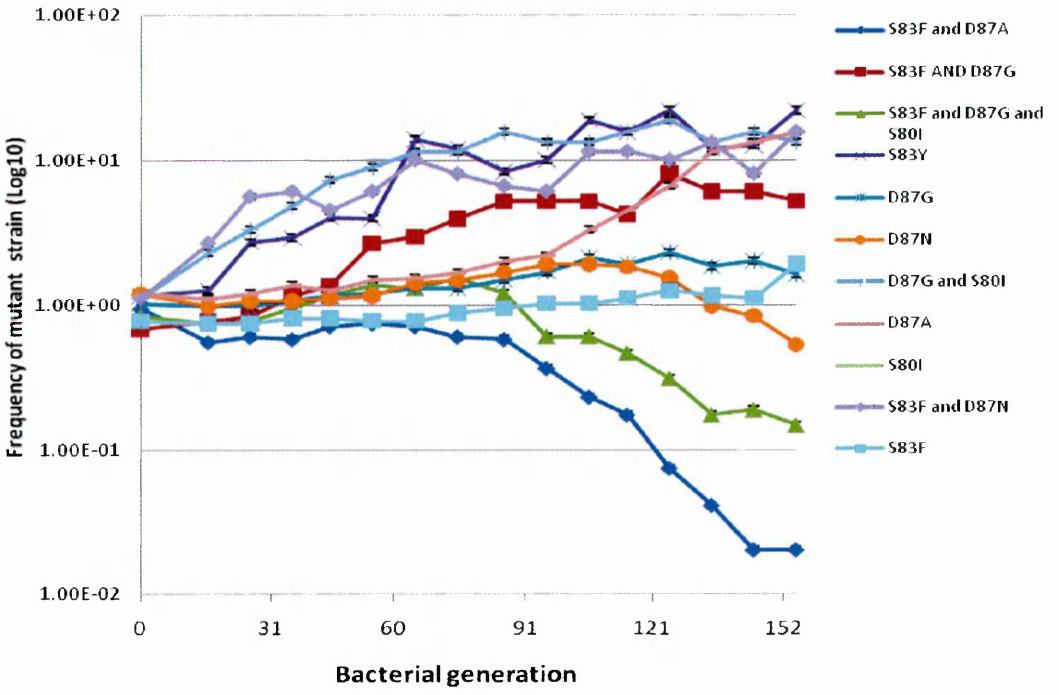


Figure 5.12 Plots of non - mutant / mutant ratios as detected by pyrosequencing from competitive growth assays

All strains were mixed in a 1:1 ratio and sub-cultured daily for 15 days. Bacterial generations were assessed by calculating the number of doubling events from the initial inoculation and the final stationary phase concentration of bacteria. The frequency of resistance was calculated by measuring specific SNP proportions in the mixed culture. Each coloured line represents the frequency of a different strain with respect to the parent strain over time.

The pyrosequencing assays were found to have a reproducible trend for all ten strains. This was shown by comparing the medians of three independent assays at the beginning and end of the experiment with the individual assays sampled at each time point (Figure 5.12). Over the 150 generations, five strains demonstrated a selective advantage over the parental strain including WT 21, WT 43, WT 141, WT 287 and WT 297, two strains had a slight selective advantage (WT 26 and WT 144), one was unchanged (WT 37) and two had a selective disadvantage (WT 300 and WT 286). The linear regression for all strains was calculated and all but one strain WT 37 (D87N) were significant, this strain demonstrated the smallest variation from a relative fitness of 1 (Table 5.4). The largest selective advantages were seen with strains WT 21 (S83Y), WT 287 (D87G and S80I) and WT 297 (D87A) which all demonstrated relative fitness greater than 1. The lowest relative fitness was seen with strains WT 300 (S83F and D87A) and the triple mutant WT 286 (S83F, D87G and S80I).

Whilst demonstrating similar growth rates as grown independently in two media, the supportive medium (LB aro) and the LB aro supplemented with 8 % oxgall medium, almost all the reconstructed mutants outcompeted with the *S.* Typhi BRD 948 strain. However, two of these *S.* Typhi mutants including the double mutant WT 300 (S83F and D87A) and the triple mutant WT 286 (S83F, D87G and S80I) demonstrated disadvantaged growth in competition with the *S.* Typhi BRD 948 strain.

5.3 Discussion

Although the mutations related to quinolone resistance and reduced susceptibility to fluoroquinolones have been extensively detected in clinical isolates [162,219,220,223,226,232-234], the genetic background of such clinical isolates is poorly defined. Here, the mutants carrying defined quinolone resistant mutations were

reconstructed in an isogenic background of quinolone susceptible BRD 948, so it was assured that all the mutants had an identical genetic background and any phenotypic differences could only be dependent on the introduced mutation(s). These reconstructed mutants were investigated for their levels of quinolone resistance and reduced susceptibility to fluoroquinolones, their rate of killing over the time by ofloxacin, their growth rates and finally their biological costs in competition with the parental *S.* Typhi BRD 948 strain.

The reconstructed mutants were shown to influence fluoroquonolone resistance in a laboratory *S.* Typhi strain, however any comparison between wild type *S.* Typhi isolates and reconstructed *S.* Typhi mutants is inappropriate since their genetic backgrounds are neither comparable nor known. It was observed that the bactericidal trend of ofloxacin to the WT141 (S83F D87G; ofloxacin MIC 0.38 μg / mL) was substantially different from that of the same substitution mutant, DT 18, which was isolated clinically from Viet Nam (ofloxacin MIC 2 μg / mL) (Figure 4.14g). The clinical *S.* Typhi strain was more resistant to ofloxacin than the reconstructed *S.* Typhi strain. Similarly, the double mutation *S.* Typhi isolate, AG 152 (OFX MIC 3 μg / mL) showed a better survival ability under prolonged ofloxacin exposure, compared to the same double reconstructed mutant WT 43 (S83FD87N; ofloxacin MIC 0.38 μg / mL), (Figure 4. 14h). Thus, it would be worthwhile to look at other fluoroquinolone resistance mechanisms since the result suggested that more than one mechanism is involved in the ofloxacin resistance phenotype in *S.* Typhi isolates.

5.3.1 Nalidixic acid resistance and reduced susceptibility to fluoroquinolones of the reconstructed *S.* Typhi mutants

Our study confirmed the hypothesis that point mutations in *gyrA* gene caused nalidixic acid resistance and reduced susceptibility to fluoroquinolones in *S.* Typhi strain, while the mutation in *parC* alone was not related to (fluoro)quinolone resistance. Our study was supported by previous data that was also conducted based on the reconstructed mutations in *gyrA* and *parC* genes of *S.* Typhi strains, but differing amino acids were substituted, and several new, additional mutations were compared [131].

5.3.2 The impact of the amino acid substitutions on MIC and corresponding bactericidal activity of the (fluoro)quinolones

Although all reconstructed mutants were resistant to nalidixic acid, the level of nalidixic acid resistance depended on amino acid substitutions. Our findings clearly demonstrated that even though the *gyrA* mutations were not the primary mechanism of fluoroquinolone resistance, they caused reduced susceptibilities to fluoroquinolones and the level of fluoroquinolone resistance depended on the amino acid substitutions and the antimicrobial used. The amino acids substituted lead to the alteration of the chemical characteristics of the enzyme active site [84]. This alternative protein formation inhibits the formation of the antimicrobial-enzyme complex. As a consequence, the antimicrobial cannot achieve its full bactericidal capacity.

5.3.3 The role of the triple mutation on fluoroquinolone resistance

Among the *gyrA* mutants, it seems that the combination of the substitution S83F with the D87G depicted the most (fluoro)quinolone resistance phenotype. However, high fluoroquinolone resistance required a combination of the double *gyrA* (S83F D87G) and one *parC* (S80I) mutations. Previous studies which were performed with an *S.* Typhi

strain [131] and an *E. coli* strain [239] demonstrated similar results; that the combination of *gyrA* and *parC* substitutions play a role in the fluoroquinolone resistance mechanism. However in these studies, different amino acids were described, in particular the combination of S83F (or Y) and D87N in *gyrA* gene with E84K in *parC* gene in the *S.* Typhi study [131] or a combination of S83L and D87G (*gyrA*) and S80I (*parC*) in the *E. coli* study [239]. The fluoroquinolone resistance rate of the triple mutant showed that the MIC of ciprofloxacin was lower (1000-fold) when compared with those of gatifloxacin and ofloxacin; 250-fold and 340-fold, respectively. Similarly, Turner *et al.* also found that the MIC of ciprofloxacin increased 4000-fold compared with 500-fold of ofloxacin and 188-fold of gatifloxacin [131]. Additionally, the triple mutant showed a similar growth rate to the parental *S.* Typhi strain and other mutants in nutrient LB aro, but slightly slower in LB supplemented with 8 % oxgall after four hours. In the competition assay with the parental BRD 948 strain, the triple mutant represented the largest growth disadvantage after 15 days (155 generations). Although representing weaker competing ability with the parental strain, under pressure of high concentration of ofloxacin (1x to 16x MIC), the triple mutant, WT 286 was completely resistant to this antimicrobial. Thus, although demonstrating a fluoroquinolone resistant *S.* Typhi strain with low biological cost when growing alone, the triple mutant had the lowest selective advantage in the competitive assay. It is a known phenomenon that antimicrobial resistant organisms generally have a selective disadvantage compared to their sensitive counterparts [243-246]. Therefore, this result is unsurprising as resistance is combined with a substantial fitness cost, a caveat that potentially prevents such organisms undergoing a dominant clonal expansion. These data suggest why, potentially, this triple mutant was only found in a small number of clinical isolates due to the negative fitness cost, yet high level resistance.

5.3.4 The role of *parC* mutation on fluoroquinolone resistance

The *parC* mutation (S80I) alone had no effect on (fluoro)quinolone resistance phenotype, this finding supported by a previous study [118,239]. Our observations have added further weight to the hypothesis that the topoisomerase IV is a secondary target for fluoroquinolones [247] and strengthened by not detecting a *parC* mutant in clinical *S.* Typhi isolates [131,162,220,226,232,233,239]. The fluoroquinolone resistant phenotype could only be detected when *parC* mutations combined with at least one *gyrA* mutation [239]. In fact, this study showed that apart from the triple mutant, the complement of *parC* mutation to the single *gyrA* mutation D87G of the strain WT 287 (D87G (*gyrA*) and S80I (*parC*)) prolonged its survival in 8x and 16x MIC of ofloxacin compared with the single D87G substitution (WT 144). The role of *parC* mutation in the biological cost of the mutants was unclear since the combination of S80I with other *gyrA* mutations did not influence the selective advantage in the competition environment.

5.3.5 The bactericidal efficacy of ofloxacin concentrations on the single and double *S.* Typhi mutants

The single and double substitutions, although mostly expressing stronger survival compared with the parental BRD 948, showed no significant difference in the time - kill of ofloxacin amongst the single and double substitutions. As a result, the 4x MIC of ofloxacin used in clinical typhoid treatment needs to be reevaluated as the majority of the mutants, except the WT 304 (S80I), WT 37 (D87N) did not demonstrate total cell killing after 24 hours exposure to ofloxacin.

Perhaps the most alarming and interesting result of this work is the competitive advantage shown by seven of ten reconstructed mutants tested. As acknowledged

above, antimicrobial resistant organisms often have a natural selective disadvantage in the absence of antimicrobials. Here we found the opposite, demonstrating that these reconstructed mutants actually have a selective advantage in the presence and absence of fluoroquinolones. These data tentatively explain the abundance of the single S83 *gyrA* mutants that were detected in the majority of nalidixic acid resistant clinical isolates. The spread of nalidixic acid resistant *S.* Typhi organisms has been shown by Roumagnac and Holt [69,71]. These studies demonstrated the clonal expansion of certain haplotypes (specifically the H58 haplotype) suggesting a further selective advantage on the basis of corresponding nalidixic acid resistant strains. However, it was assumed that this occurred due to the overuse and constant exposure of organisms to low level of fluoroquinolones. Our findings suggest that such organisms have a natural selective advantage and the use of such antimicrobials may have forced these organisms through a bottleneck and permitted rapid clonal expansion. These findings require further studies to ascertain and understand how such antimicrobial resistant pathogenic organisms are selected and spread through human populations.

The evolution of a fluoroquinolone resistant *S.* Typhi strain is not only dependent on the mutation in the target genes, but is also influenced by compensatory mechanism, host adaption, antimicrobial usage and even living environment. Although our study has only contributed the roles of specific *gyrA* and *parC* resistance mechanisms in *S.* Typhi strains, these data are an important foundation for further investigations on the development and dissemination of *S.* Typhi in susceptible populations.

6. Development of the Multiplex Ligation dependent-Probes Amplification (MLPA) for *S.* Typhi isolates in Asian countries

6.1 Introduction

S. Typhi is a human restricted bacterial pathogen and studies of the populations of the organisms (both local and global) have shown limited variation [248]. Genotyping of bacterial populations is important to study evolution and the dissemination of the organism in question. Genotyping *S.* Typhi has been performed by several methods, including non DNA sequence based and DNA sequence based techniques. None sequence based approaches have included ribotyping, plasmid typing, phage typing, Random Amplified Polymorphic DNA (RAPD-PCR) [70,249-251]. Lately, Pulsed Field Gel Electrophoresis (PFGE) was the 'gold standard' method for *S.* Typhi genotyping although the results produced by PFGE only have limited discriminatory power [252-254]. The development of the PCR based Amplified Fragment Length Polymorphism (AFLP) method permitted better resolution and had a higher discriminatory power for strain differentiation when compared to PFGE [255]. The downside to all these aforementioned techniques is a lack of reproducibility and understandable phylogeny.

The only useful marker applicable for performing molecular epidemiology is DNA sequence variation. In 2002, Kidgell *et al.* performed the first sequence based phylogenetic study on *S.* Typhi [68]. This multi locus sequence typing (MLST) of a global collection of *S.* Typhi organisms found only limited sequence diversity and because of this limited diversity MLST was unsuitable for a universal typing scheme. The MLST study paved the way for a more intense investigation of nucleotide variation in the *S.* Typhi population.

A robust genotyping of *S.* Typhi was provided only by the development of mass DNA sequencing based techniques. These methods have been shown to improve discriminatory ability in genotyping a highly clonal (monophyletic) pathogen like *S.* Typhi. Currently Single Nucleotide Polymorphism (SNP) typing is the most robust methodology for genotyping *S.* Typhi [69,71,256]. Based on the results of SNP detection in 200 gene fragments on the *S.* Typhi chromosome, fifty-nine haplotypes were found to be circulating globally and haplotype H58 was the most prevalent in Viet Nam and southern Asia. Four additional haplotypes (H1, H50, H58 and H63) have also found to be circulating in Viet Nam [71]. However, H58 predominates and is associated with the Ser83 nalidixic acid resistance mutation in the *gyrA* gene [71]. Additionally, in Viet Nam, Le *et al.* demonstrated the occurrence of microevolution within an *S.* Typhi clone [257]; and even though independent outbreaks of multi - drug resistant typhoid fever in southern Viet Nam were demonstrated to be caused by single bacterial strain, different outbreaks were not caused by a single *S.* Typhi strain [41]. The stimulus behind the regional spread of fluoroquinolone resistant *Salmonella* belonging to genotype H58 is still unknown, although data presented here suggests a selective advantage of these strains even in the absence of fluoroquinolone usage [71].

SNP typing has been developed and is currently based on investigating the nature of some 1,700 differing SNP loci in *S.* Typhi isolates, these data provide a fully resolved phylogenetic tree which allows one to trace the genetic changes that occurred during clonal diversification [258]. Whilst the SNP method is the current 'gold standard' for such bacterial populations these techniques are somewhat labor intensive and expensive, as they require sophisticated equipment. Therefore, SNP typing cannot directly be applied to studying *S.* Typhi populations in locations where *S.* Typhi is endemic. We wished to develop a simple universal methodology that could be used to

perform genotyping in a basic molecular laboratory in a developing country. However, to understand the phylogeny of the *S.* Typhi populations in these settings, this new methodology needed to be based upon the current phylogenetic data from SNP typing and genome sequencing. To achieve such a typing method we selected Multiplex Ligation Dependent-Probes Amplification (MLPA) to detect specific chromosomal markers. MLPA was first applied to investigate host genetic polymorphisms and is a relatively simple and robust multiplex assay which allows simultaneous genotyping and, in our case, the detection of drug resistance mutations [156]. In order to develop and validate an appropriate method for genotyping nalidixic acid resistant *S.* Typhi isolates in endemic countries, we generated an MLPA assay to study our observed *gyrA* mutations and phylogenetic insertion and deletion fragments in the *S.* Typhi chromosome (Figure 6.1) [69]. To ensure we could detect the major haplotypes, this method was applied in comparison with the conventional Golden Gate SNP typing technique.

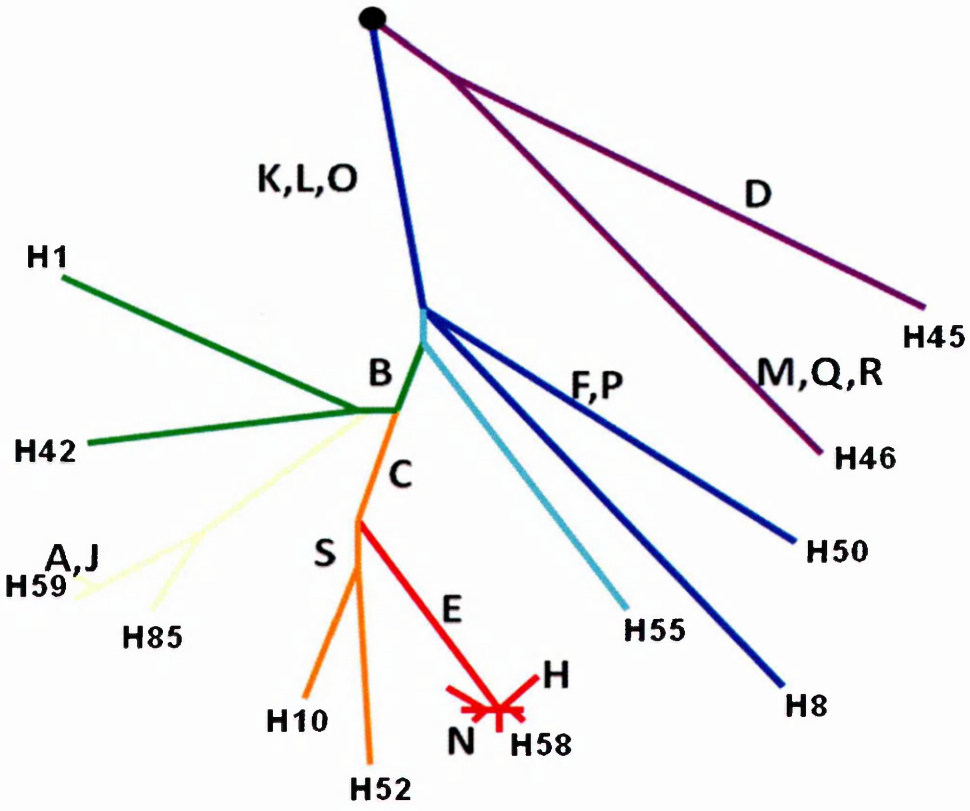


Figure 6.1 Haplotype spanning tree based on SNP typing and showing insertion and deletions in the *S.* Typhi population

The phylogenetic insertion – deletion tree was provided by Holt [69]. Branch colours indicate different lineages of *S.* Typhi haplotype; Branch lengths were differentiated by the representative deletions. The block capital represents the deletion / insertion fragment.

6.2 Results

Seventy three *S.* Typhi strains isolated between 1994 and 2006 and representative of strains from seven Asian countries were selected for genotyping (Table 2.5). These strains were examined previously in Chapter 4 and any mutations in their *gyrA* or *parC* genes had been identified. These strains were initially subjected to discrimination by the Illumina GoldenGate SNP typing assay at the Wellcome Trust Sanger Institute in Cambridge, United Kingdom and by the MLPA assay which was designed in house to detect 16 distinguishable fragment lengths as section 2.8.2.

6.2.1 *S.* Typhi SNP typing by the Golden Gate assay

In this study, genotyping was firstly performed by a SNP interrogation using the GoldenGate system [259]. This assay not only detects specific SNPs at each specific locus but also can detect SNPs which are demonstrative of insertions, deletions and plasmid sequences. From 73 representative strains from Bangladesh, China, India, Indonesia, Laos, Pakistan and Viet Nam selected for genotyping (Table 2.5) seven haplotypes were detected. In total, 72.6 % (53 / 73) of *S.* Typhi strains observed were of the H58 haplotype. The H58 haplotype was detected in 5 Asian countries including Bangladesh, India, Laos, Pakistan and Viet Nam. In Viet Nam, 4 / 45 *S.* Typhi strains belonged to the H1 haplotype and the remaining 41 strains belonged to the H58 group. Haplotype H58 was detected in 4 / 5 strains from Bangladesh; the remaining strain was H42, the only isolated belonging to this haplotype. Four from five of the *S.* Typhi strains from India belonged to group H58, the other Indian *S.* Typhi strain was haplotype H50. Three haplotypes were detected from Laos and were H1, H5 and H58. Three haplotypes were also detected in *S.* Typhi strains from Pakistan, these were H42, H50 and H58 (Table 6.1).

Haplotype H58 is currently the dominant strain circulating in southern Asia [71]. However, H58 was not detected in China or Indonesia. Amongst the *S.* Typhi strains isolated from China, 3 / 5 belonged to haplotype H52 and 2 / 5 strains belonged to group H50. While 1 / 3 of the *S.* Typhi strains and 2 / 3 strains from Indonesia belonged to haplotypes H50 and H59, respectively (Table 6.1).

Table 6.1 The *S.* Typhi haplotypes found in seven Asian countries

Seventy three *S.* Typhi strains including 45 Vietnamese strains, 5 Bangladeshi strains, 5 Chinese strains, 5 Indian strains, 3 Indonesian strains, 5 Laotian strains and 5 Pakistani strains were subjected to SNP detection using the GoldenGate method.

Country	Year	No of strain (n = 73)	Genotype (7)
Viet Nam	1994 - 2004	4	H1
		41	H58
Bangladesh	2003	1	H42
		4	H58
China	2002	2	H50
		3	H52
India	2003	1	H50
		4	H58
Indonesia	2002	1	H50
		2	H59
Laos	2003	2	H1
		2	H52
		1	H58
Pakistan	2002	1	H42
		1	H50
		3	H58

We hypothesized that *S.* Typhi haplotype may be related to the multidrug resistance phenotype. Therefore, we interpreted the haplotype discrimination amongst 71 *S.* Typhi strains in the correlation with the MDR phenotypes which were presented in Chapter 3. Forty six percent (33 / 71) MDR *S.* Typhi were detected to belong to haplotypes H1 (1

strain), H42 (1 strain) and H58 (30 strain), while other 53.5 % (38 / 71) non - MDR *S.* Typhi were found to belong to haplotypes H1 (3 strains), H42 (2 strain), H50 (5 strains), H52 (5 strains), H58 (20 strains), H59 (2 strains) and H85 (1 strain). Thus, the MDR phenotype was defined only in haplotypes H1, H42 and H58.

A phylogenetic tree of 92 *S.* Typhi strains including 73 *S.* Typhi strains from the Asian collection and 19 *S.* Typhi control strains was constructed based on SNP database (Section 2.8.1) (Figure 6.2). To generate the tree, the phylip file of SNP database was analyzed using the RAxML program. The maximum-likelihood phylogeny was analyzed using a GTR substitution model. The maximum-likelihood was preferred since it showed fast handling missing data well and the GTR model provided the best fit to this data using model test. The phylogenetic tree was drawn using Dendroscops version 2.3 based on the *S.* Typhi SNPs database [260].

We analyzed the genetic variation of *S.* Typhi by interpreting the phylogenetic tree (Figure 6.2). We divided the *S.* Typhi strains into the 7 main polymorphic groups. Approximately 67 % (62 / 92) *S.* Typhi strains accounted for phylogenetic group 6. These strains included the Bangladeshi, Indian, Moroccan (the control strain ISP-03-7467), Pakistani strains which were isolated in 2003 - 2004 and Vietnamese strains which were isolated in 1994 – 2004. Approximately 32 % (29 / 92) *S.* Typhi strains accounted for other 6 genetic groups including group 1 to group 5 and group 7. The control *S.* Typhi E-00-7866 strain accounted for group 1. Group 2 comprised 6 strains including 2 Chinese strains, 1 Indonesian strain, 1 Pakistani strain and 2 *S.* Typhi control strains (E-98-3139 and M 223). Phylogenetic group 3 included an Indian strain and a Kenyan control strain (E-98-0664). Whilst, phylogenetic group 4 included 14 strains, in which 5 Indonesian strain (2 *S.* Typhi isolates and 2 control strains), 2

Pakistani strains, 2 Laotian strains, and 5 Vietnamese strains were involved, and phylogenetic group 7 included 2 strains from Laos and 2 control strains (Ty2 and E-01-6750).

Thus, H58 haplotype was spanned in Asian countries in the years 2003 and 2004. Notably, almost all *S.* Typhi isolated from Viet Nam during 13 years (1994-2006) belonged to H58 haplotype. The phylogenetic tree based on SNP typing demonstrated that although *S.* Typhi strains in Asian countries was closely related they were divided into seven sub-groups, in which most of strains from Viet Nam represented a unique group.

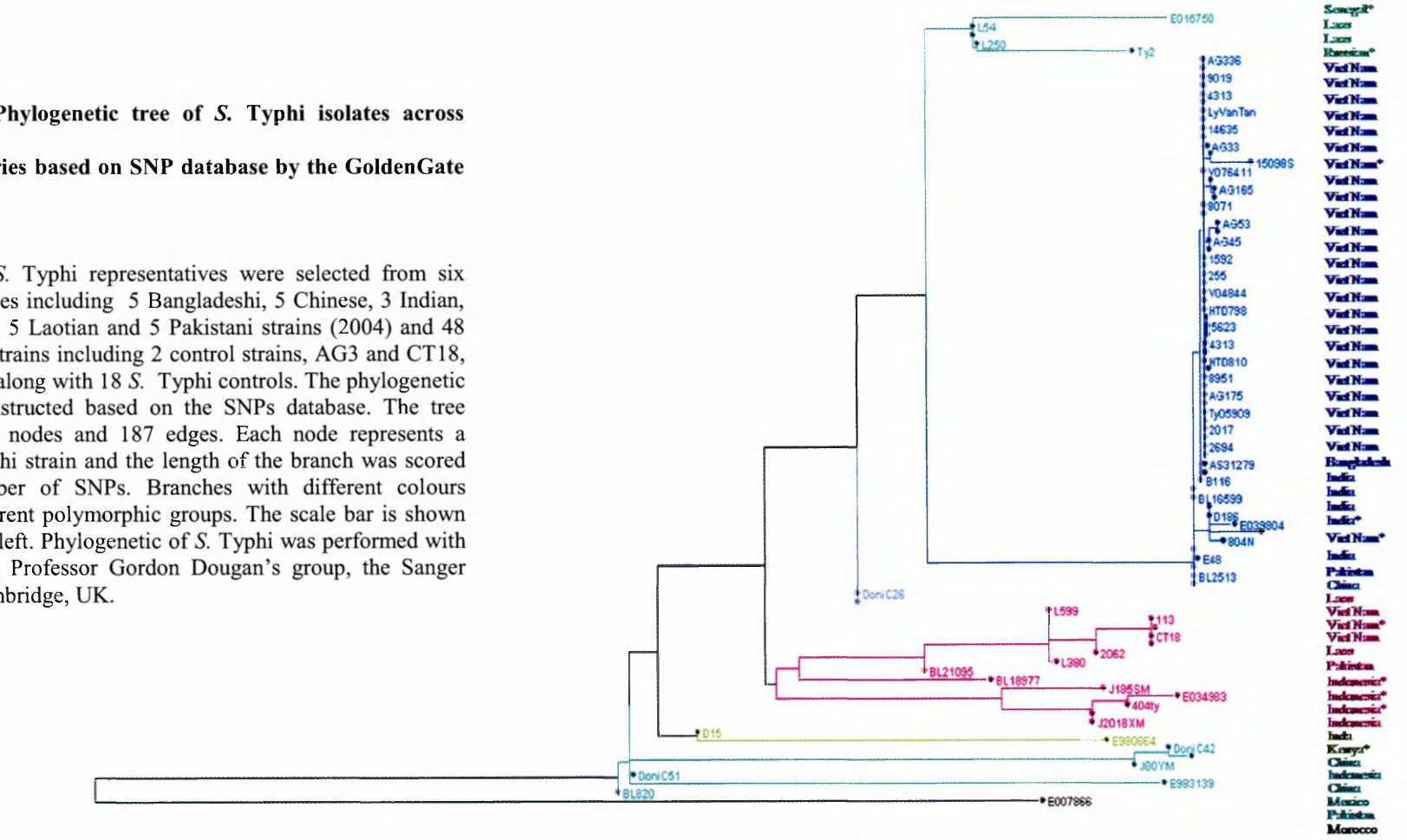


Figure 6.2 Phylogenetic tree of *S.* Typhi isolates across Asian countries based on SNP database by the GoldenGate assay

Ninety two *S.* Typhi representatives were selected from six Asian countries including 5 Bangladeshi, 5 Chinese, 3 Indian, 3 Indonesian, 5 Laotian and 5 Pakistani strains (2004) and 48 Vietnamese strains including 2 control strains, AG3 and CT18, (1994-2006) along with 18 *S.* Typhi controls. The phylogenetic tree was constructed based on the SNPs database. The tree includes 188 nodes and 187 edges. Each node represents a single *S.* Typhi strain and the length of the branch was scored by the number of SNPs. Branches with different colours indicate different polymorphic groups. The scale bar is shown at the higher left. Phylogenetic of *S.* Typhi was performed with support from Professor Gordon Dougan's group, the Sanger Institute, Cambridge, UK.

6.2.2 Developing the Multiplex Ligation Dependent - Probes method for *S.* Typhi genotyping

We included 18 *S.* Typhi strains which had been previously haplotyped with defined insertion - deletion profiles as controls for MLPA genotyping. Sixteen-probe pairs were designed in a mastermix based on 11 insertion - deletion fragments discovered by Holt *et al.* [69], including two pairs of probes detecting changes at position 83 and 87 of the *gyrA* and position 80 of the *parC* genes of *S.* Typhi strain and three probe pairs worked as internal controls for *S.* Typhi species (section 2.8.2).

The MLPA results were visualized by the automated ABI sequencer in which the results were analyzed using the Gene Mapper software version 4.0 (Figure 6.3). In addition the conventional agarose gel electrophoresis was used as an alternative method for fragment visualization (Figure 6.4).

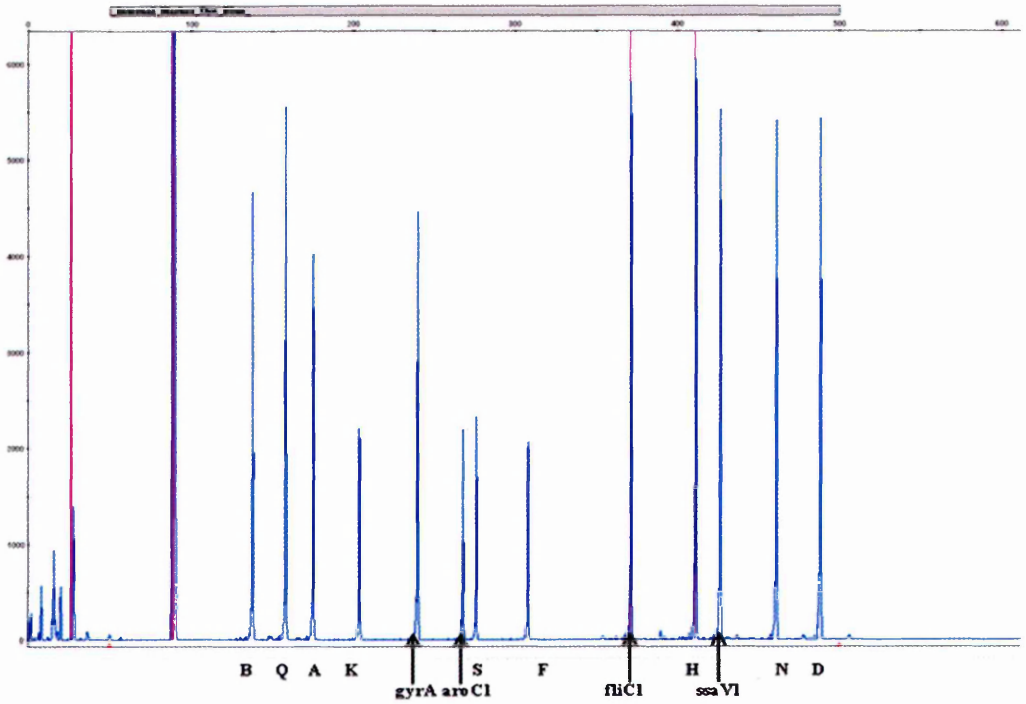


Figure 6.3 Representative of fragment length analysis of the multiplex ligation-dependent probe amplification (MLPA)

Thirteen peaks of the Pakistani *S.* Typhi strain BL 3723 depict the presences of 6 representative deletion markers including Q (158 bp), A (175 bp), S (275 bp), F (308 bp), H (414 bp), N (465 bp) and D (494 bp); two common deletions including B (139 bp) and K (204 bp) which were always depicted peaks. A *gyrA* peak (238 bp) for detecting mutation(s) and three *S.* Typhi controls including aroC1 (267 bp), fliC1 (374 bp) and ssaV1 (429 bp) are indicated by arrows.

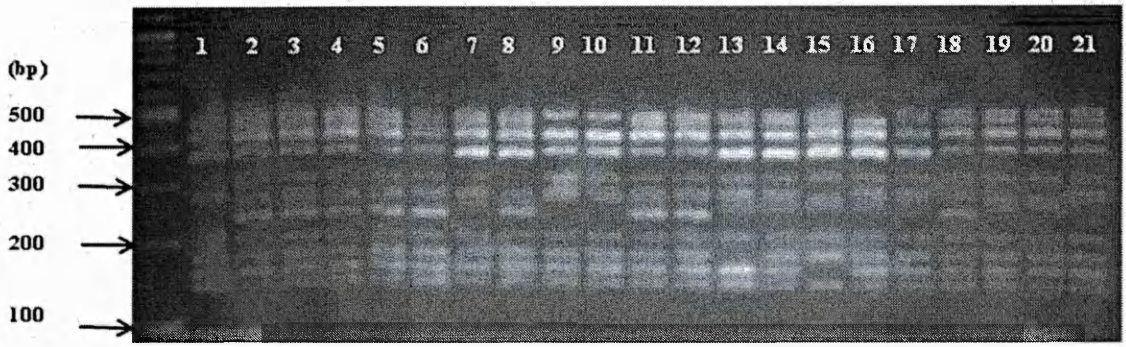


Figure 6.4 The representative fragment length analysis by agarose electrophoresis

Each lane depicts an *S.* Typhi strain including 15 *S.* Typhi haplotype controls including the E98-2068 (Lane 1), E98-0664 (Lane 2), E98-3139 (Lane 3), Ty2 (Lane 4), 404TY (Lane 5), E02 - 1180 (Lane 6), E00 - 7866 (Lane 7), E03 - 9804 (Lane 8), ISP - 03 - 07467 (Lane 9), ISP - 04 - 06979 (Lane 10), E02 - 2759 (Lane 11), E03 - 4983 (Lane 12), 150 (98) S (Lane 13), 8(04)N (Lane 14) and M 223 (Lane 15); and six *S.* Typhi isolates including DoniC 4 (Lane 16), J 2018 (Lane 17), BL 2513 (Lane 18), BL 820 (Lane 19), AG 3 (Lane 20) and DoniC 42 (Lane 21). Each band either represents an insertion fragment, a *gyrA* / *parC* mutation or the internal controls. Fourteen bands include Q (158 bp), A (175 bp), S (275 bp), F (308 bp), H (414 bp), N (465 bp) and D (494 bp); two deletions including B (139 bp) and K (204 bp); *gyrA* or *parC* mutations (130 bp and 238 bp, respectively). Three *S.* Typhi control fragments include the aroC1 (267 bp), fliC1 (374 bp) and ssaV1 (429 bp).

By MLPA, we could define 5 genetic polymorphic groups (A, B, F, E and S). Group A was haplotype H59, group B included H1, H42 and H85, group F was haplotype H50, group E represented haplotype H58, group K included H8 and H55 and group S includes haplotypes H10 and H52.

The genotyping results achieved by MLPA were confirmed by the SNP typing results which were represented in Table 6.2.

Table 6.2 Haplotype comparisons between SNP typing and MLPA of the *S.* Typhi strains in Asian countries

Sixty eight representative *S.* Typhi strains including 5 strains from Bangladesh, 5 strains from China, three strains from India, three strains from Indonesia , 4 strains from Laos, 5 strains from Pakistan and 43 strains from Vietnamese collections and 18 haplotyped *S.* Typhi control strains were subjected for genotyping comparison. The deletions were depicted in capital letters according to Holt *et al.* [69] and MLPA profile was named according to the deletion representative of each haplotype group.
(*) Deletion profile was based on SNP typing database.

Country	Year	No of strain (n = 68)	Deletion profile*	MLPA profile	Genotype (6)
Viet Nam	1994 - 2004	3	K,B	B	H1
		40	B,K,C,E	E	H58
Bangladesh	2004	1	K,B	B	H42
		4	B,K,C,E	E	H58
China	2004	2	K,F	F	H50
		3	K,B,C,S	S	H52
India	2004	1	K,F	F	H50
		2	B,K,C,E	E	H58
Indonesia	2004	1	K,F	F	H50
		2	K,B,A	A	H59
Laos	2004	1	K,B	B	H1
		2	K,B,C,S	S	H52
		1	B,K,C,E	E	H58
Pakistan	2004	1	K,B	B	H42
		1	K,F	F	H50
		3	B,K,C,E	E	H58

6.2.3 The relationship between mutations conferred nalidixic acid resistance and *S.* Typhi haplotype

Mutations in *gyrA* conferring nalidixic acid resistance could be detected in *S.* Typhi isolates by MLPA method. Amongst 55 *S.* Typhi strains detected to be harbored *gyrA* mutation(s), 50 strains were haplotype H58 and approximately half of the strains (26 / 50) represented MDR phenotype haplotype. No *parC* mutation was detected by MLPA technique.

By designing specific primer pairs to directly detect the deletions from genomic DNA, we could demonstrate the presence or absence of these deletion fragments in the *S.* Typhi collection. However, the PCR condition for each specific primer pair was not similar; therefore PCR conditions were established separately for each assay. The deletions were defined either by the presence of a shortened length of a band or the absent of any band. The results achieved by conventional PCR were identical to the MLPA results.

6.3 Discussion

6.3.1 *S.* Typhi genotyping in Asian countries

This study compared a large collection of geographically diverse *S.* Typhi strains collected in typhoid endemic regions. Amongst 7 haplotypes identified in this study, H58 were predominant and found in 5 / 7 Asian countries.

Our findings are supported by a previous report which demonstrated the circulation of several *S.* Typhi haplotypes in Asian countries [71,256]. By performing SNP analysis on 105 global *S.* Typhi collection, Roumagnac *et al.* showed the presence of haplotypes H45, H57 and H60-65 in Asian countries along with haplotypes H1, H42, H50, H52, H58 and H85, while the result here demonstrated the presence of H59 in Indonesian *S.* Typhi strains along with haplotypes H1, H42, H50, H52, H58 and H85 [71]. The presence of haplotype H59 in Indonesian *S.* Typhi strains was also reported previously [73]. Additionally, 3 out of 18 Asian *S.* Typhi strains analyzed in this study were also described by Roumagnac *et al.*, however this study yielded different results compared by SNP typing analysis, instead of belonging to haplotype H65, H74 and H73 [71], our SNPs data divided 3 strains (E48 (India), DoniC51 (China), AS 18977 (Bangladesh)) into haplotypes H58, H50 and H42, respectively.

A study conducted in a province of northern Viet Nam showed that *S.* Typhi strains were genetically related [177]. Similarly, we proved that almost all of the *S.* Typhi strains belonged to H58 genotype even though these *S.* Typhi strains were isolated from different outbreaks in the south of Viet Nam over a 12 year period (1994-2005). By genotyping *S.* Typhi strains from 4 different outbreaks in Viet Nam from 1993-1997, Connerton *et al.* showed that multidrug-resistant typhoid fever in southern Viet Nam was caused by single bacterial strains and different outbreaks were not clonally related [41]. This point of view was supported by a another study which showed multiple clones of *S.* Typhi circulating in the population [261]. Furthermore, microevolution and differences in gene content within a clone was also demonstrated [257,262] and it is probable that the clonality of an organism depends on the relative concept of each study setting [263].

Further investigations of the presence of mutations conferring fluoroquinolone resistance have shown that 89.3 % *S.* Typhi strains of haplotype H58 carried *gyrA* mutation and 52 % of these *S.* Typhi were MDR. Moreover, the co-persistence of H58 haplotype *S.* Typhi strains with *gyrA* mutations delivering fluoroquinolone resistance with diversity in multidrug resistant phenotypes was continuously detected in Viet Nam for 15 years (1994-2006). Thus, the MDR phenotype may not account for the emergence of H58 *S.* Typhi clone which carries *gyrA* mutation(s) throughout Asian countries. There are several different hypotheses for this phenomenon. Our study seemingly supports the idea that the extensive use of fluoroquinolones in typhoid therapy is responsible for the dissemination of fluoroquinolone resistant *S.* Typhi strains along with the low biological cost which might enable this genotype to spread widely. Yet, the persistence of H58 with *gyrA* mutation(s) implies that the advantage of this clone is not only due to antimicrobial selective pressure but also to host and

environmental adaptations. The H58 *S.* Typhi clone maybe able to survive persistently in asymptomatic carriers, and can therefore, transmit to others via a oral - fecal route and it may be able to cause infection under specific advantageous conditions. However, the spread of this clone would not solely be affected by those components. Other influential factors could also be mutation, recombination, gene acquisition [73], genetic drift [69], the loss of functional genes or rearrangement in the genetic structure [264,265]. Moreover, the combination of haplotype and phenotype would provide a considerable insight into the genetic structure of the *S.* Typhi population. This study may be considered limited due to the small size of *S.* Typhi strains compared. Therefore, to study the genetic diversity of *S.* Typhi, to boost the understanding of evolution of *S.* Typhi a large sample collection is required.

6.3.2 MLPA typing

The recent advances of high throughput DNA detection technologies, such as dHPLC and whole genome approaches (the 454 and the Solexa sequencing), have greatly facilitated genotyping of high conserved bacterial pathogens [69,71,256]. Such techniques have revealed greater information on the evolution of *S.* Typhi. There has been a lack of a transferrable technique which permits the genotyping of *S.* Typhi in typhoid endemic countries. Here we aimed to design and validate an optimum methodology for *S.* Typhi genotyping in such settings. The correlation between the genetic background and FQ resistance phenotype could not be interpreted by SNP typing since the specific *gyrA* mutations could not be designed on the GoldenGate platform. MLPA appeared to be a promising technique, as this assay can detect both haplotypes and resistance phenotype. Using MLPA, the *gyrA* and *parC* mutations can visualized along with other genomic markers, but the exact nucleotide substitution needs to be confirmed by sequencing. Compared with other typing techniques,

evolutionary information of the organisms can be shown by MLPA. Additionally, this approach allows visualizing deletion patterns both by agarose electrophoresis and fragment length analysis. Thus, this technique can be flexibly transferred in different setting and the data gained can be transposed from laboratory to laboratory. This method also demonstrated advantages in terms of cost effectiveness, when compared with other high throughput methodologies. Although requiring some technical training, the MLPA method is relatively straight forward to perform and the results achieved are reproducible and reliable. Furthermore, for rapid screening of haplotypes and *gyrA* and *parC* mutations in *S.* Typhi isolates, MLPA is an ideal alternative and appropriate for epidemiological surveillance in developing countries.

7. General discussion

This work investigated the antimicrobial resistance patterns of 2,028 *S.* Typhi isolates in 8 typhoid endemic regions including Bangladesh, China, Indonesia, India (2003, 2006), Pakistan, Laos, Nepal and Viet Nam (1994-2005). The results have contributed to the knowledge of current antimicrobial resistant typhoid in endemic countries. As discussed in Chapter 3, the burden of typhoid caused by antimicrobial resistant *S.* Typhi was significant in 5 countries including Bangladesh, India, Nepal, Pakistan and Viet Nam. In contrast, typhoid caused by resistant *S.* Typhi strains was substantially less in Indonesia, China and Laos. In fact, in this study, no MDR *S.* Typhi strains were identified in Indonesia and China and no NAR *S.* Typhi strains were detected in Indonesia and Laos. MDR *S.* Typhi strains with additional nalidixic acid resistance and reduced susceptibility to fluoroquinolones were prevalent in other countries. However, azithromycin resistance was only detected in Chinese and Indian *S.* Typhi strains and low level of ceftriaxone resistance was solely found in Nepalese *S.* Typhi strains. The distinctive antimicrobial resistance profiles in China and Indonesia were supported by molecular genotyping investigations. The most predominant genotype (H58), which is associated with nalidixic acid resistance and reduced susceptibility to fluoroquinolones, was identified in Bangladesh, India, Pakistan and Viet Nam, and none of the *S.* Typhi strains isolated in China and Indonesia were of the H58 haplotype. It is evident that specific haplotypes with different resistance phenotypes circulate in specific locations despite the overall dominance of the H58 genotype.

The MDR phenotype was found to be persistent in Viet Nam over the last 13 years; conversely there was a decrease in MDR strains found in India between 2003 and 2006. It has been postulated that the loss of MDR phenotype is a result of a sequential sub-culturing *in vitro*, since plasmids may not be stable in *S.* Typhi because of defects in the

partition of new plasmids in new bacterial cells [266]. Additionally, a shift in the use of first line antimicrobials in typhoid treatment to fluoroquinolones has also lead to the loss of plasmids in *S.* Typhi strains due to the removal of selective pressure caused by antimicrobial treatment [266]. Further evidence suggests that there may be a decrease in typhoid fever caused by MDR organisms in some areas [127,189,267]. The reversion to sensitivity to older antimicrobial may allow for alternative therapies, however, these patterns may only be short lived.

The burden of antimicrobial resistance in *S.* Typhi was demonstrated across several Asian countries, our data suggests that Viet Nam was a hot spot of both MDR and nalidixic acid resistant and reduced susceptibility to fluoroquinolones problem over the last 13 years. As reported in 2008, 70.8 % of the population of southern Viet Nam is rural and Ho Chi Minh City is the most crowded area in the country, with the population density of 3,155 person / Km^2 [268]. The infrastructure of this area has not grown at the same pace as the population or personal economic wealth. Therefore, there is a lag in providing clean water, as well as hygiene and sanitation improvements. Typhoid fever disease was demonstrated to be significant in school-age children and young adults in Mekong Delta provinces, these findings indicate poor sanitation in this region [42]. Health care services in rural areas of Viet Nam, particularly in the south are inadequate to support a large number of residents (personal information). Regardless of socioeconomics, outbreaks of MDR typhoid fever in the last decade in Mekong River Delta may have also been influenced by geographical and environmental characteristics. The river density and annual flooding for 2 - 4 months during the wet season may enhance *S.* Typhi transmission in the region.

A second aim of this study was to investigate the molecular mechanisms of fluoroquinolone resistant *S.* Typhi isolated in endemic countries. Seven types of chromosomal mutations were found in 462 *S.* Typhi isolates. These mutations were demonstrated to be significantly related to fluoroquinolone resistant phenotypes, in which the substitution of Phe for Ser at codon 83 of the *gyrA* gene was dominant. High level fluoroquinolone resistance was demonstrated to correlate with the triple mutation (S83F and D87G (*gyrA* gene) and S80I (*parC* gene)). Although resistance phenotypes were associated with *gyrA* and *parC* mutants, the impact of these substitutions was unknown. To demonstrate the influence of *gyrA* and *parC* mutations on the fluoroquinolone resistance phenotypes, these chromosomal mutations were re-generated in *S.* Typhi BRD 948. The fluoroquinolone susceptibilities of these mutants showed that at least one mutation at codon 83 or / and 87 of the *gyrA* gene were the key to nalidixic acid resistance and reduced susceptibility to fluoroquinolones and the *parC* mutation alone did not cause resistance. The level of resistance was dependant on the amino acid substitution, the locus where mutation occurred and the generation of fluoroquinolones. The triple mutant had a highest level of fluoroquinolone resistance, yet had an increased biological cost during independent growth and a selective disadvantage in direct competition with the parental BRD 948 *S.* Typhi strain. Moreover, the S83F mutant, the most prevalent fluoroquinolone resistant mutant observed in wild type *S.* Typhi strains, was demonstrated to have a substantial selective advantage in the competition assay. These data suggest a reasonable explanation for the spread of this S83F *S.* Typhi mutant in Asian countries.

Antimicrobial resistance has caused increasing concern in the management of not only typhoid but also other bacterial infections. Antimicrobial resistance can be transmitted to offspring vertically (such as chromosomal mutations) or horizontally (such as MDR

plasmids). Making the investigation of molecular mechanisms of antimicrobial resistance is crucial in a wide variety of organisms. Studying the biological cost of bacteria would not be comprehensive without placing them in the context of clinical and epidemiological features to aid the understanding of transmission dynamics in human populations. Bacterial fitness could also be related to their growth characteristics within a specific host, such as, the organism's ability to withstand environmental stresses within and between different hosts and the organism's ability to disseminate and infect new hosts [269]. Many aspects associated with bacterial biological fitness should be considered to develop a greater understanding. To this end, reconstructing competitive assays using a surrogate infection model, such *S.* Typhimurium in mice, may add greater insight into understanding of biological fitness of *S.* Typhi.

Using whole genome sequence data, the MLPA based typing method on representative deletions has been developed. This technique is appropriate for a multiple locus genotyping approach on *S.* Typhi strains and on other pathogens in endemic countries. This method generates reliable and reproducible data and is sufficiently flexible to apply in laboratory settings such that in Viet Nam. The combination of MLPA and the high throughput pyro-sequencing applications support epidemiological investigations of antimicrobial resistant pathogens. Although representative deletions applied in this study may not provide efficient information on the overall genetic relatedness of the strains, the results achieved offer the data on basic bacterial genotypes as well as resistant phenotypes providing helpful initial grounding in the genetic structure of bacterial populations in an endemic area.

The impact on human health of antimicrobial resistance *S.* Typhi infection is apparent as the disease causes increased morbidity, increased cost of treatment due to increasing

length of hospital duration and concurrent loss of earning for patients [269]. The overall management of typhoid fever requires improving drinking water sources, the upgrading of sewage systems and hygienic food; however these are all long term goals [74]. Therefore, antimicrobial therapy is still essential for typhoid in resource-poor settings [74]. It is a controversial issue for the control of typhoid fever as to what is more important, the continuous usage of fluoroquinolones, the development of new potential antimicrobials or the development and deployment of new vaccines. Vaccines against *S.* Typhi are the most applicable choice for the elimination of the disease as well as effectively controlling antimicrobial resistance. Vaccination development is suitable not only for *S.* Typhi but also for other enteric bacteria such as *S.* Paratyphi. Current typhoid vaccines are only protective against *S.* Typhi infections whereas an imbalance in the competition between enteric organisms may, potentially, cause a serotype shift from *S.* Typhi to *S.* Paratyphi [72,270]. Nevertheless, mass vaccination programmes in endemic countries have rarely been applied even though they are recommended by the World Health Organization [74].

This study has contributed to the understanding of the antimicrobial susceptibilities and the underlying molecular biology of this important enteric pathogen. Future studies may be focused not only on mechanisms of fluoroquinolone resistance but also on the abilities of the organisms to survive and spread both *in intro* and *in vivo* conditions. Effective tracking of transmission routes through asymptomatic carriers and environmental vehicles should also be investigated. Extensive molecular epidemiological surveillance using the MLPA technique, or an equivalent, should be established regionally and internationally for the purpose of understanding the circulation of *S.* Typhi organisms. This research, despite unavoidable limitations, is the first study investigating the molecular epidemiology of fluoroquinolone resistant *S.*

Typhi in Asia in general and in Viet Nam in particular, making a great effort in describing the resistance and its mechanisms and its survival features. It also provides crucial information for the public health workers on the disease prevention and control programmes as well as updating reliable and effective treatment regimens for patients.

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